PHD

Aspects of PRPP synthetase and RNA ribose in T.b.brucei

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Aspects of PRPP synthetase and RNA ribose in *T.b.brucei.*

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by

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Dedicated to my father John,

my mother Maroula

and my grandmother Kiki Potamitis

for their love and support during my student days.

'I will praise you, O Lord, among the nations;

I will sing of you among the peoples.

For great is your love, reaching to the heavens;

your faithfulness reaches to the skies.

Be exalted, O God, above the heavens;

let your glory be over all the earth.'

Psalm 57:9-11,

The Holy Bible,

New International Version.

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List of Symbols and Abbreviations.

$(Km)_{P1}$	Michaelis constant for first product.
$(Km)_{S3}$	Michaelis constant for third substrate.
(Veff)ind	maximum effective rate for indicator reaction.
1,3 DPGA	1,3' diphosphate glycerate.
1,3-DPGA	1,3'-diphosphoglycerate.
3-keto-6PG	3'-keto-6'-phosphogluconate.
3-PGA	3'-phosphoglycerate.
6PG	6'-phosphogluconate (6'-phosphogluconic acid).
ACTase	aspartate carbamoyltransferase.
Ado	adenosine.
ADP	adenosine 5'-diphosphate.
ALDO	aldolase.
AMP	adenosine 5'-monophosphate.
APP	4'-aminopyrasolopyrimidine.
APPR-DP	4'-aminopyrasolopyrimidine ribonucleotide 5'-diphosphate.
APPR-MP	4'-aminopyrasolopyrimidine ribonucleotide 5'-monophosphate.
APPR-TP	4'-aminopyrasolopyrimidine ribonucleotide 5'-triphosphate.
AS	ammonium sulphate.
AS P	ammonium sulphate pellet.
Asp	aspartic acid.
ATP	adenosine 5'-triphosphate.
BS	bloodstream.
C-Asp	carbamoylaspartate.
C-Pi	carbamoylphosphate.
Cf	*Crithidia fasciculata.*
Cl	*Crithidia luciliae.*
CNS	central nervous system.
cpm	counts per minute.
CP	cultured procyclic.
CPSase	carbamoylphosphate synthetase.
DEAE	diethylaminoethyl.
DHAP	dihydroxyacetone phosphate.
DHOase	dihydro-orotase.
DNA	deoxynucleic acid.
dpm	disintegrations per minute.
DTT	Dithiothreitol.
E	eluent.
EDTA	ethylenediamine tetraacetic acid.
Eprim	enzyme of unknown activity (primary enzyme).
Eind	enzyme of known (directly measurable) activity (indicator enzyme).
Er4P	erythrose 4'-phosphate.
F6P	fructose 6'-phosphate.
FDP	fructose 1,6'-diphosphate.

FPLC	Fast protein liquid chromatography.
g	acceleration due to gravity; 9.81 m x s^{-2}.
G-3-P	L-glycerol 3'-phosphate.
G-6-P	glucose 6'-phosphate.
G6PDH	glucose 6'-phosphate dehydrogenase.
GAP	glyceraldehyde 3'-phosphate.
GAPDH	glyceraldehyde 3'-phosphate dehydrogenase.
GDP	glucose 1,6-diphosphate.
Gln	glutamine.
GMP	guanosine 5'-phosphate.
GOD	glucose oxidase.
HEPES	4'-(2'-hydroxyethyl)piperazine-1'-propanesulphonic acid.
HGPRTase	hypoxanthine-guanine phosphoribosyltransferase.
HPLC	high pressure liquid chromatography.
HPPR-MP	allopurinol (4-hydroxypyrazolo [3,4-α] pyrimidine 5'-monophosphate.
Hx	hypoxanthine.
Hxr	inosine.
IMP	inosine 5'-phosphate.
ITP	inosine 5'-triphosphate.
Ki	dissociation constant of EI (the inhibitor constant).
Ki	equilibrium constant for dissiciation of I from EIS.
Km	concentration of substrate giving half maximal velocity (the Michaelis constant).
KRPB	Krebs-Ringer phosphate buffer.
KRPSG	Krebs-Ringer phosphate buffer with sucrose and glucose.
L	*Leishmania* spp.
µmole	micromole = 10^{-6} mole.
mmole	millimole = 10^{-3} mole.
mUnit	milliunit defined as the amount of enzyme required to produce 1 nmole of product in 1 minute of reaction.
NAD	nicotinamide adenine dinucleotide.
NADH	reduced nicotinamide adenine dinucleotide.
NADP	nicotinamide adenine dinucleotide phosphate.
NADPH	reduced nicotinamide adenine dinucleotide phosphate.
NDP	nucleotide diphosphate.
nmole	nanomole = 10^{-9} mole.
NMP	nucleotide monophosphate.
NMR	nuclear magnetic resonance.
NTP	nucleotide triphosphate.
nUnit	nanounit defined as the amount of enzyme required to produce 1 pmole of product in 1 minute of reaction.
OA	orotate.
ODCase	orotidine 5'-phosphate decarboxylase.
OMP	orotidine 5'-phosphate.
OPRTase	orotate phosphoribosyltransferase.
OTP	orotidine 5'-triphosphate.

P_1 P_2 P_3 P_4	concentration of first, second, third and fourth product respectively.
PEG	polyethylene glycol.
PEI	polyethyleneimine.
PGK	3'-phosphoglycerate kinase.
pH	Negative logarithm of hydrogen ion concentration ($-\log_{10}H$).
pK	Negatine logarithm of the dissociation constant (-logK).
Mr	molecular weight, relative molecular mass.
Pi	inorganic phosphate.
pmole	picomole = 10^{-12} mole.
PMSF	Phenylmethylsulphonyl fluoride.
POD	peroxidase.
PPi	inorganic pyrophosphate.
PPiase	inorganic pyrophosphatase.
PPP	pentose phosphate pathway.
PRPP	phosphoribosyl pyrophosphate; α-D-Ribofuranose 1-pyrophosphate 5'-phosphate; 5'-Phosphoribosyl-α-1'-pyrophosphate.
PRTase	phosphoribosyltransferase.
R1P	ribose 1'-phosphate.
R5P	ribose 5'-phosphate.
R_f	the ratio of distance moved by a component to moved by the solvent in a given chromatogram.
RNA	Ribonucleic acid.
RNAse	Ribonuclease.
Ru5P	ribulose 5'-phosphate.
S_1 S_2 S_3	substrate concentration for first, second and third substrate respectively.
S7P	sedoheptulose 7'-phosphate.
SHAM	salicylhydroxamic acid.
SiC	silicon carbide.
spp.	species.
SSC buffer	Sodium chloride and sodium citrate buffer.
TA	transaldolase.
Tb	*Trypanosoma brucei.*
Tc	*Trypanosoma cruzi.*
TCA	tricarboxylic acid.
TCA	Trichloroacetic acid.
TDR	Training in Tropical Diseases.
TE buffer	Tris-HCl and EDTA buffer.
TEMED	tetramethylene diamine.
TIM	triosephosphate isomerase.
TK	transketolase.
TLC	Thin Layer chromatography.
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride.
Trizma	Tris(hydroxymethyl)aminomethane.
TTP	thymidine 5'-triphosphate.
U.V.	ultra violet.

UMP	uridine 5'-phosphate.
UPRTase	uracil phosphoribosyltransferase.
UrKase	uridine kinase.
URPTase	uracil phosphoribosyltransferase.
VAT	variable antigen type.
Vind	maximum rate of indicator enzyme reaction.
Vmax	maximum rate of enzyme reaction.
Vprim	maximum rate of primary reaction.
VSG	variant specific glycoprotein.
WHO	World Health Organisation.
XMP	xanthosine 5'-phosphate.
Xu5P	xylulose 5'-phosphate.

Abstract.

Experiments were designed to confirm the existence in bloodstream forms of *T.b.brucei* of a small, but significant, proportion of supplied glucose being metabolised through an oxidative pentose phosphate pathway leading to provision of ribose-5-phosphate which is subsequently incorporated into RNA. The experiments also confirmed the existence of some *de novo* synthesis of pyrimidine bases, but not purines.

Phosphoribosyl pyrophosphate (PRPP) is the essential substrate for supply of the ribose moiety in the biosynthesis of purine and pyrimidine nucleotides. It occupies a unique position connecting the major metabolic pathways of the pentose phosphate shunt and nucleotide synthesis.

Phosphoribosylpyrophosphate synthetase (ATP:D-ribose-5'-phosphate pyrophosphotransferase, PRPP synthetase,EC 2.7.6.1) is the sole enzyme responsible for the formation of PRPP and so its presence was investigated in bloodstream forms of *T.brucei*. The enzyme was detected in bloodstream forms and the cultured procyclic forms of *T.brucei* by an adapted form of assay measuring the PRPP-conversion of [8-^{14}C] Hypoxanthine to [^{14}C] 5'-IMP in the presence of hypoxanthine-guanine phosphoribosyltransferase. The specific activity of the enzyme in the crude extracts of bloodstream forms was calculated to be 0.125 nmoles PRPP formed/min/mg cell protein.

The enzyme from the bloodstream form was partially purified and characterised and its properties compared to those PRPP synthetases from other sources.

The partially purified enzyme was found to have a Michaelis constant for D-ribose-5'-phosphate of 0.027mM. $MgATP^{2-}$ acts as an inhibitor at high substrate concentrations, with Km of 0.063mM and Ki of 0.250mM. Although inorganic phosphate did not seem significantly to stabilise the enzyme during storage at 4°C, glycerol at 20% (v/v) concentration increased its stability with time. There was no activity with other triphosphates except dATP which gave 75% of the activity with ATP.

Gel filtration studies showed that the partially purified enzyme has a molecular weight of ~180,000. Using subcellular fractions provided by Dr. F. R. Opperdoes, PRPP synthetase activity was found mainly in the cytosolic fraction, albeit with a sedimentation behaviour that indicated a very high molecular weight. This dichotomy was resolved when it was found that the molecular weight of the enzyme in crude extracts, prepared similarly to that used for the localisation study, was in excess of 600,000. The formation of high molecular weight aggregates is characteristic of PRPP synthetase from other sources.

CHAPTER 1

INTRODUCTION TO TRYPANOSOMES

1.0. General introduction.

The word 'parasite', derived from ancient Greek, means one frequenting the tables of the rich and earning welcome by flattery. It includes examples of all the known infectious agents such as viruses, bacteria, fungi, protozoa and helminths, which at some time during their life cycle intimately associate with the infected host, even though the host may not be flattered and the infectious agents may not be exactly welcome (Wang, 1983). In further contrast to the above definition, the parasites of most medical importance are sitting at the tables of the poor and causing 3 billion people malnutrition, blindness, debility, disfiguration, and death in the tropics and subtropics and to domesticated animals all over the world. The cost of parasites in terms of human misery and economic loss is incalculable.

The medical importance of trypanosomiases and leishmaniases is emphasised by the inclusion of African and South American trypanosomiases and leishmaniases as two target diseases of the World Health Organisation Programme for the Research and Training in Tropical Diseases (TDR) sponsored by the World Bank and the United Nations Development Programme (Trigg, 1979) . For both diseases vector control is either very expensive or impractical and drugs are either toxic or unavailable since pharmaceutical companies turned their backs on these diseases as potential sources of revenue (Molyneux and Ashford, 1983).

1.1. Introduction to trypanosomes.

Trypanosomes are parasitic protozoa that cause several serious diseases of humans and domestic animals in tropical Africa and South America. The family Trypanosomatidae consists of a great variety of genera that infect birds, mammals (*Trypanosoma, Leishmania*), fish, amphibia, insects (*Crithidia* spp.), and even plants. Many have complex life cycles that may comprise both extracellular and intracellular forms. Transmission to the vertebrate host always takes place *via* blood-sucking vectors, such as flies, bugs, fleas, or leeches. The

classification of the genus *Trypanosoma* is shown in figure 1.1. The two main groups of the genus reflect the mode of transmission by the insect vector, the Stercoraria (faecal transmission) and the Salivaria (salivary transmission).

Over the last two decades these parasitic protozoans have attracted much attention because they combine a number of peculiarities. The five main structural features characteristic of trypanosomes are shown in figure 1.2 and are:
(1) a single flagellum,
(2) a complex skeletal array of subpellicular microtubules,
(3) glycosomes,
(4) a single mitochondrion associated with
(5) a dense network of DNA called kinetoplast (Fairlamb, 1982).

The forms in the life cycle of the kinetoplastid flagellates are brought about by the migration of the kinetoplast-flagellum complex within the body (Vickerman, 1976), as shown in figure 1.3. The leishmanial parasites possess only two forms in their life cycles; amastigotes in the cells of the lymphoid macrophage system of a mammal or lizard and promastigotes in the gut of the vector which is a sand-fly (Diptera, Phlebotomidae). *Leishmania* species cause serious diseases in man. The typical infection is cutaneous but in many species, the parasites may invade subcutaneous or deeper tissues causing hideous and permanent disfiguration. The most serious disease, Kala azar, involves the macrophages of organs such as liver. The main species of *Leishmania* that affect man are *L.mexicana, L.braziliensis, L.tropica* and *L.donovani* (Cox, 1982).

1.2. African trypanosomes.

Salivarian trypanosomes cause African sleeping sickness in humans (*T.b.rhodesiense* and *T.b.gambiense*), nagana in cattle (*T.b.brucei, T.b.vivax, T.b.congolense*), surra in horses and camels (*T.b.evansi*) and dourine in horses (*T.b.equiperdum*).

Recent estimates suggest that 45 million people are exposed to the risk of infection in Africa. Until 1979 there were some 10,000 new cases reported each

CLASSIFICATION OF TRYPANOSOMES

KINGDOM: Animalia

SUB-KINGDOM: Protozoa

PHYLUM: Sarcomastigophora

CLASS: Zoomastigophora

ORDER: Kinetoplastida

GENUS: Trypanosoma

SECTION 1: Salivaria

SUB-GENUS: Trypanozoon

SPECIES: *Trypanosoma brucei brucei*

Trypanosoma brucei rhodesiense

Trypanosoma brucei gambiense

for example.

SECTION 2: Stercoraria

SUB-GENUS: Schizotrypanum

SPECIES: *Trypanosoma cruzi.*

Figure 1.1. The classification of Trypanosomes (according to Cox, 1982 ; Molyneux and Ashford, 1983).

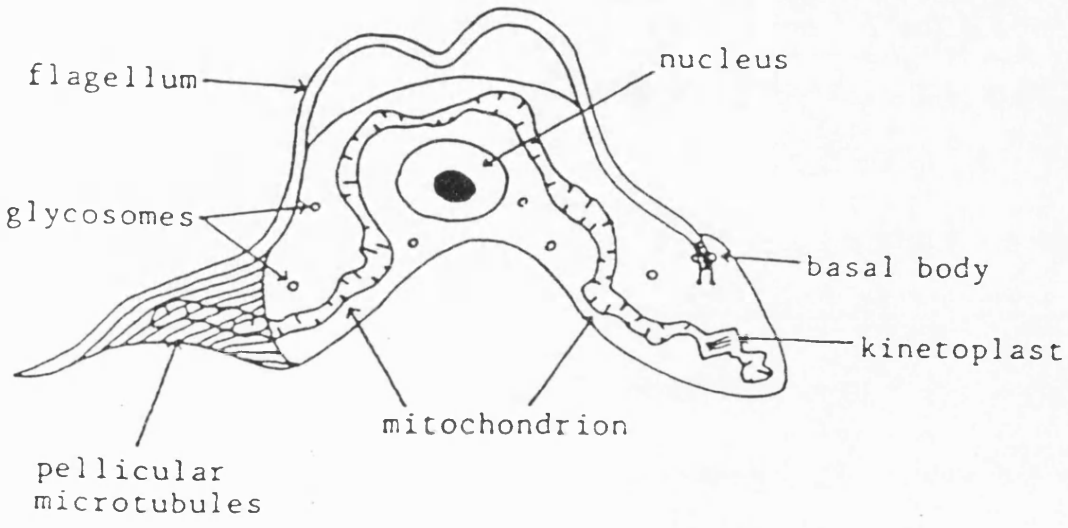


Figure 1.2. The gross morphology of *T.b.brucei* (intermediate bloodstream form, Vickerman, 1965) , showing the five main structural features.

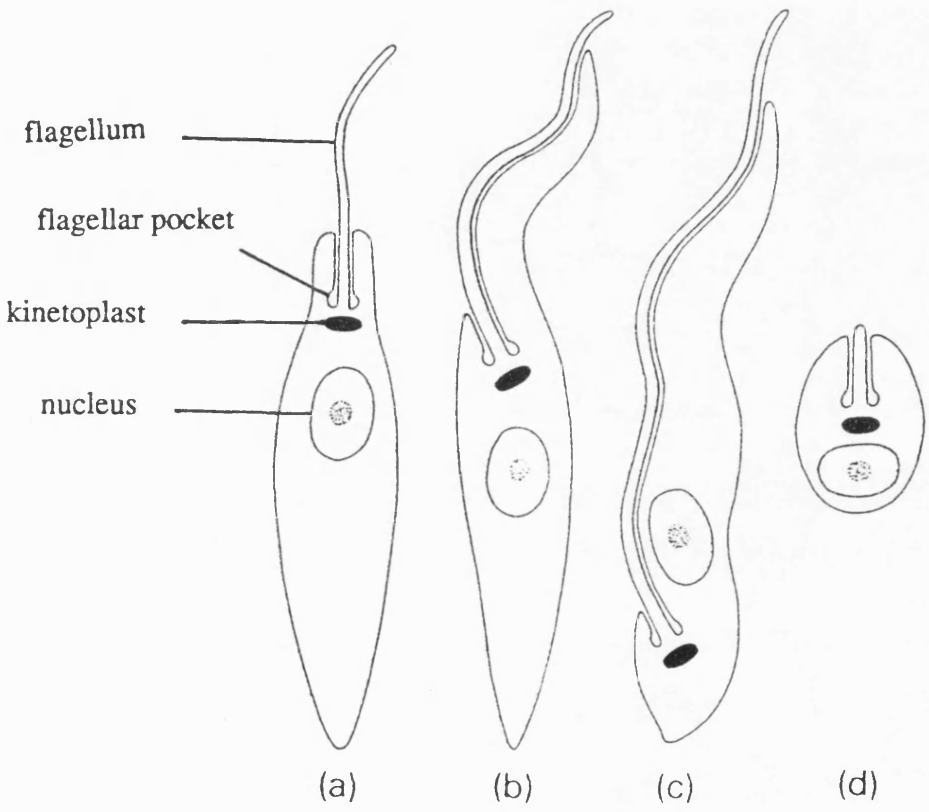


Figure 1.3. Different forms of trypanosomes according to kinetoplast-nucleus orientation (after Vickerman, 1976).

(a) = promastigote

(b) = epimastigote

(c) = trypomastigote

(d) = amastigote.

year (De Raadt, 1976) , but serious outbreaks in Cameroon, the Sudan and Uganda have increased the annual incidence to at least 20,000 (Goodwin, 1985). The 1985 Ugandan epidemic caused by the *rhodesiense* form of the disease and the outbreaks in the Southern Sudan since the 1970s of the *gambiense* form illustrate the profound effects of the breakdown of diagnostic and surveillance facilities brought about through civil unrest.

The three forms of the Trypanozoon subgenera, *T.b.rhodesiense, T.b.gambiense* and *T.b.brucei*, are thought to be identical both morphologically and biochemically (Hoare, 1964), but some biochemical differences may exist, particularly in their infectivity to man; *T.b.brucei* being unable to survive in the human bloodstream due to a component of human serum, thought to be a high-density lipoprotein, which causes this trypanosome to lyse (Rifkin, 1978) . Infection with *T.b.gambiense* gives rise to the chronic form of the disease while an infection with *T.b.rhodesiense* follows a more acute course (Wakelin, 1984).

The first sign of the acute form of sleeping sickness is a swollen, rubbery chancre at the site of infection or frequently a more general oedema of the face. Some weeks following infection a fever develops, irregular and intermittent in nature, more intense in the acute form of the disease. Other symptoms of general malaise include headache, pains of the joints, loss of weight, insomnia and anaemia. It is the invasion and destruction of the central nervous system that makes the disease invariably fatal. Invasion of the central nervous system is accompanied by epileptic fits, loss of apetite, and eventual death due to respiratory failure, altered heart rhythm and beat or by secondary infections caused by the immunosuppressive activity of the trypanosomes.

Animal trypanosomiasis (nagana) is a serious obstacle to human welfare, because it causes severe nutritional and economic problems. Over three million cattle die each year from various forms of the disease, and the rearing of domestic cattle, sheep and goats is impossible in 10 million square kilometers of Africa which is the habitat of the tsetse fly (*Glossina* spp.).

1.3. South American trypanosomes.

The most important South American trypanosome is *T.cruzi*. At least 20 million people in South America are infected with *T.cruzi*; in Brazil alone the annual incidence of new cases of Chagas' disease is estimated at 120,000. Young children are particularly likely to die in the acute febrile stage, whereas adults tend to survive to the chronic phase of the disease, in which parasite damage to the heart muscle causes congestive heart failure and autonomic nervous tissue damage causes dilatation of the oesophagus and colon. Other stercorarian parasites of importance include, *T.lewisi* of rats, *T.theileri* of cattle and *T.melophagium* of sheep.

1.4. Life cycle of *T.b.brucei*.

T.b.brucei is cyclically transmitted from mammal to mammal by the insect vector, the tsetse fly, during feeding (see figure 1.4).

Different developmental stages of the life cycle have markedly different morphology and biochemical activity. When an infected tsetse fly bites an uninfected human or animal, metacyclic forms are injected with the fly's salivary secretions. These forms develop into trypomastigote forms and migrate to the bloodstream. Bloodstream trypomastigotes show considerable variation in morphology (pleomorphism), ranging from slender to stumpy forms. Stumpy forms are non-dividing and are thought to be a preadaptation necessary for survival in the insect midgut. After a blood meal from an infected animal, short stumpy forms develop into procyclic trypomastigotes which ultimately migrate to the fly's salivary glands to complete the cycle (Fairlamb, 1982).

1.5. Life cycle of *T.cruzi*.

T.cruzi is transmitted by reduviid bugs. Development in the insect vector takes place in the gut, the infective trypomastigote form occurring in the hind gut. Transmission of these parasites to vertebrates takes place when infected faeces of the vector come into contact with broken skin or mucous membranes,

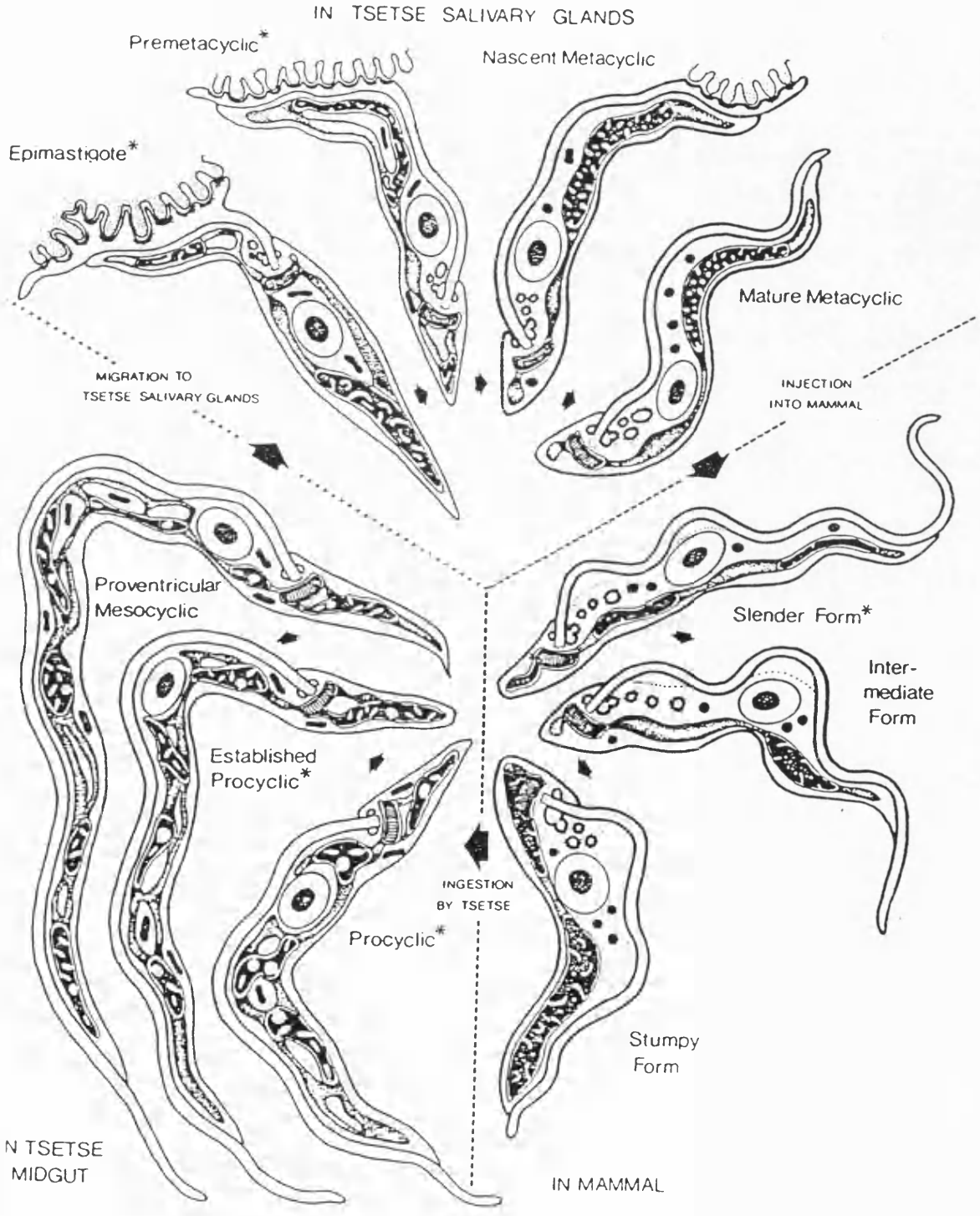


Figure 1.4. The life cycle of *T.b.brucei*, with emphasis on the relative sizes of the various stages. The forms marked with an asterisk are those in which division occurs (Vickerman, 1985).

particularly of the eye or by blood transfusions (Fairlamb, 1982). These non-dividing forms either penetrate new host cells, particularly muscle, or are ingested during a blood meal by an uninfected reduviid bug. The life cycle of *T.cruzi* is shown in Figure 1.5.

1.6. The morphology of trypanosomes.

The cyclically transmitted trypomastigotes which occur in the bloodstream and tissues of infected animals and man vary widely in size and shape, from short stumpy, about 12μm long, to long slender, about 30μm long. The trypanosomes are surrounded by a plasma membrane or plasmalemma approximately 2-4nm in total width, similar in appearance to that of other animal cells. Underlying the plasma membrane there is a series of pellicular microtubules which are believed to be of cytoskeletal function and present in both the vertebrate and invertebrate forms of *Trypanosoma* and *Leishmania* (Mólyneux and Ashford, 1983). Ultrastructural studies suggest that desmosome-like structures attach the plasma membrane to the flagellar membrane (figure 1.2) and sliding of the flagellar microtubules seem to cause the plasma membrane to undulate (Smyth, 1976).

An unusual feature of African trypanosomes is the possession of a single mitochondrion which undergoes striking changes during the various stages of the life-cycle. The mammalian form of trypanosome contains a simple mitochondrion which is unbranched with few tubular cristae, while the insect form has a fully-developed mitochondrion with numerous plate-like cristae (Vickerman, 1965; Fairlamb, 1982). Within the single mitochondrion, a characteristic organelle, the kinetoplast, is located. It is made up of a complex network of interlocked circular DNA molecules, representing up to 30% of the total cellular DNA (Opperdoes, 1985).

In the mammal, all trypanosomes are invested with a compact, homogeneous layer of identical glycoprotein molecules, the surface coat, 12-15nm thick and comprising up to 10 million molecules per cell (Vickerman,

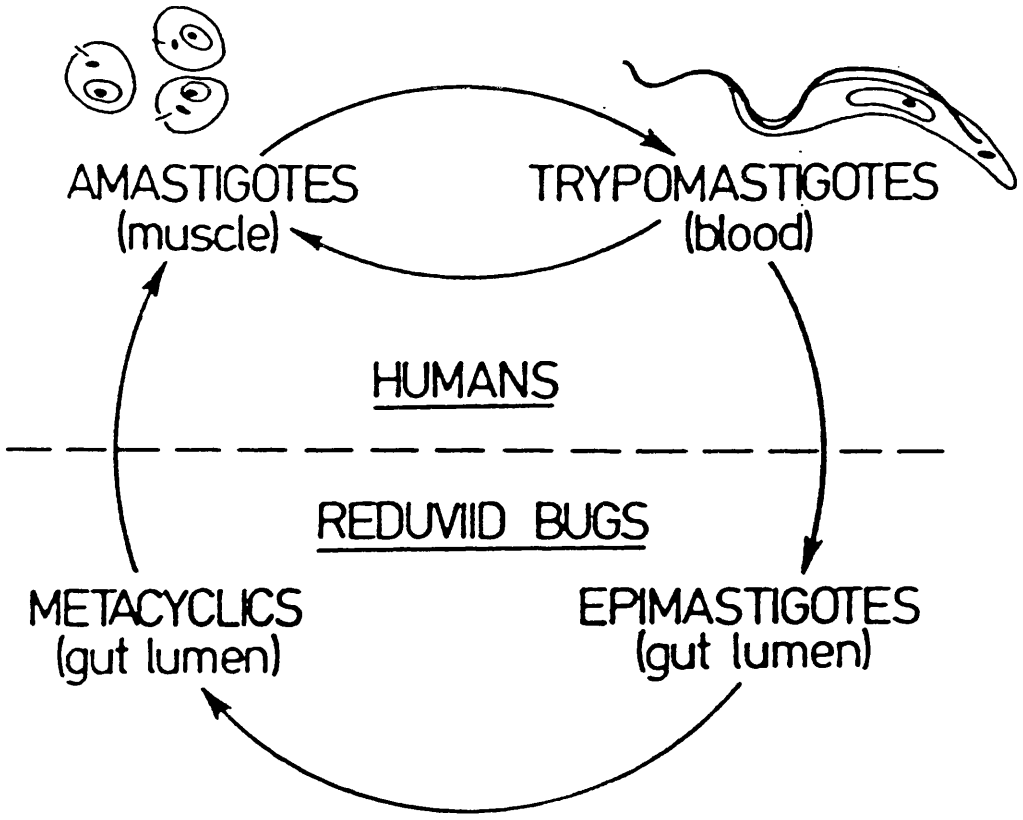


Figure 1.5. The life cycle of *T.cruzi* (Fairlamb, 1982).

1969; Whitfield, 1979). The 52-69 kDa glycoprotein comprising this coat stimulates a powerful immune response on the part of the host, which is evaded by switching from expression of one surface glycoprotein to expression of another. Antigenic change entails the exposure of new epitopes on the N-terminal portion of the glycoprotein polypeptide. When the trypanosomes are injected by the fly into the host the majority possess glycoprotein coats of a single kind, the homotype. Trypanosomes belonging to one variable antigen type (VAT) express the same variant-specific glycoprotein (VSG) gene. A minority of this population however have antigenically-different coats or heterotypes by VSG switching. The immune system of the host quickly responds to the homotype by raising IgM-type antibodies which results in agglutination and complement-mediated lysis of the homotype (Cox,1982). Sacrifice of the homotype allows a heterotype to grow up to take its place in the next parasitaemic peak characteristic of the undulating parasitaemia of trypanosome infections. A single *T.brucei* cell has a repertoir of VATs based on about a thousand VSG genes which represents between five and ten percent of the total genetic capacity of the trypanosome (Donelson and Turner, 1985).

1.7. The Glycosome.

Glycosomes are microbody-like organelles unique to all of the Trypanosomatidae with no mammalian counterpart (Opperdoes, 1988).They are homogeneous in size, with an average diameter of 0.27μm. A normal bilayer membrane, which is 6-7nm thick, surrounds the contents of the glycosome. On average a bloodstream trypanosome contains 240 glycosomes, which represent between 4.3% (Opperdoes *et al.*, 1984) and 8% (Bohringer and Hecker, 1975) of its total volume and a similar percentage of its protein content (Misset *et al.*, 1986; Opperdoes *et al.*, 1984). Seventy percent of the glycosomal protein has been found in the matrix, whereas the remainder seems to be associated with the membrane (Opperdoes *et al.*, 1984). There are a limited number of polypeptides which are associated with the bloodstream-form glycosome; the major poly-

peptides have all been identified as the subunits of the glycolytic enzymes, which together account for more than 87% of the glycosome's total protein (Aman *et al.*, 1985; Misset, *et al.* , 1986). In the procyclic stage the relative abundance of the glycosomal polypeptides differs significantly, and a few additional polypeptide bands have been found (Hart *et al.* , 1984). The total amount of polypeptides is still very limited.

The glycosome contains several enzymes of glycolysis and glycerol metabolism as well as enzymes involved in such diverse pathways as carbon dioxide fixation, pyrimidine biosynthesis, ether-lipid biosynthesis, and purine salvage (Figure 1.6, Opperdoes, 1990) .

Only trace amounts of DNA were detected in glycosomal fractions, and there has been no indication of a glycosome-specific class of DNA (Opperdoes *et al.* , 1984). Current evidence suggests that glycosomal proteins are encoded in the nucleus and synthesised on free polysomes (Hart *et al.* , 1987). Import into the glycosomes occurs within minutes of synthesis without apparent cleavage of a signal peptide or other post-translational modification (Hart *et al.* , 1987). No single unifying signal for import of glycosomal enzymes has been identified, although Wierenga *et al.* (1987) proposed that the 'hot spots' characteristic of those enzymes, might serve as topogenic signals for import into the glycosome. These 'hot spots' are two localised net positively charged clusters of amino acids, approximately 40 Å apart, found on the surface of the proteins.

Opperdoes and Borst (1977) suggested that the glycosomal membrane acts like a selective permeability barrier to the bulk of the phosphorylated glycolytic intermediates and proposed that specific translocaters for DHAP, G-3-P, PGA, and Pi facilitate their diffusion over the membrane. An alternative explanation is that the single unit membrane of the glycosome acts as a simple diffusional barrier to both metabolites and coenzymes as well as maintaining a locally high concentration of glycolytic enzymes to account for the high glycolytic rate observed (Opperdoes, 1987; Aman and Wang, 1986).

Pathway	Enzyme	Organism*
Glycolysis	Hexokinase	Tb, Tc, L, Cl
	Phosphoglucose isomerase	Tb L, Cl
	Phosphofructokinase	Tb L, Cl
	Aldolase	Tb L, Cl
	Triosephosphate isomerase	Tb L, Cl
	Glyceraldehyde-phosphate dehydrogenase	Tb L, Cl
	Phosphoglycerate kinase	Tb L, Cl
Glycerol metabolism	Glycerol-3-phosphate dehydrogenase	Tb L, Cl
	Glycerol kinase	Tb L,
CO_2 fixation	Phosphoenolpyruvate carboxykinase	Tb, Tc, L, Cl
	Malate dehydrogenase	Tb
Pyrimidine synthesis	Orotate phosphoribosyltransferase	Tb, Tc, L, Cf
	Orotidine-5′-phosphate decarboxylase	Tb, Tc, L, Cf
Purine salvage	Hypoxanthine guanine phosphoribosyl transferase	Tb, Tc, L
Etherlipid synthesis	DHAP acyltransferase	Tb L
	Acyl/alkyl DHAP reductase	Tb
	Acyl-CoA reductase	Tb
Oxidation of fatty acids	Palmitoyl-CoA synthetase	L
	β-Hydroxybutyrate-CoA dehydrogenase	L
Others	Adenylate kinase	Tb L
	Catalase	Cl
	Phosphomannose isomerase	Tb

*Tb. *T. brucei*; Tc, *T. cruzi*; L, *Leishmania* spp; Cl, *Crithidia luciliae*; Cf, *Crithidia fasciculata*.

Figure 1.6. Enzymes biochemically localised in glycosomes of the Trypanosomatidae (Opperdoes, 1990).

1.8. The glycolytic pathway in trypanosomes.

The changes in morphology seen during the lifecycle of the trypanosome are associated with its ability to adapt its respiratory pathway to the prevailing conditions of its environment.

Owing to the absence of a functional mitochondrion, the bloodstream form of *T.brucei* is entirely dependent on glycolysis for its production of energy (Grant and Fulton, 1957) . Because this form lacks significant polysaccharide reserves or high-energy phosphate stores such as creatine phosphate or polyphosphates, it relies entirely on an exogenous source of carbohydrate (Opperdoes *et al.* , 1976b). Glucose is the preferred energy source but fructose, mannose and glycerol can also support motility and respiration (Ryley, 1962).

The African trypanosome, as exemplified by *T.brucei*, has an extremely high rate of glycolysis (85 nmole glucose are consumed per minute per milligram cell protein). Under an aerobiosis, one glucose molecule can generate only two ATP molecules, with two pyruvate molecules excreted into the host bloodstream as the only end product (Grant and Sargent, 1960; Fairlamb and Bowman, 1980). This low energy yield makes it necessary to have such a high glycolytic rate (Brohn and Clarkson, 1980). Von Brand (1951) calculated that the amount of glucose consumed was equivalent to 50-100% of the dry weight of a cell per hour. One mole of oxygen is utilised per mole of glucose (Ryley, 1956) and experiments with [^{14}C]glucose have shown that 83% of the glucose carbon can be recovered as pyruvate and 9% as glycerol (Grant and Fulton, 1957).

The high glycolytic rate, 50 times that in the mammalian host, is made possible not only by the abundant glucose supply in the host blood (approximately 1mg per ml, 5mM; Whitfield, 1979), but also by the clustering of most of the glycolytic enzymes in the glycosomes of the parasite (Opperdoes and Borst, 1977).

After the trypanosome's transformation from bloodstream form to insect stage its consumption of glucose occurs at a much lower rate (22.5 nmol glucose per minute per milligram of protein) (Bienen *et al.* , 1983) . The transition is

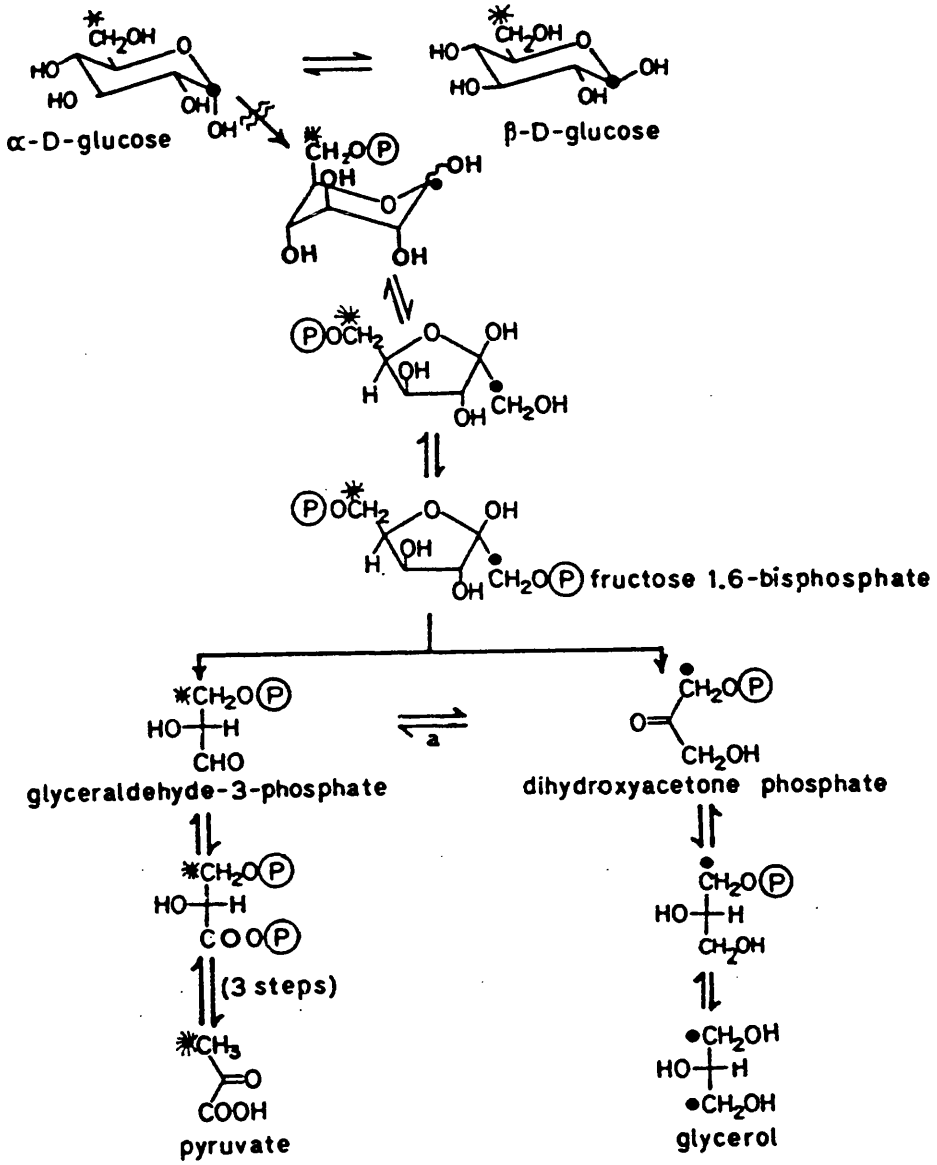
accompanied by the synthesis of certain enzymes of the tricarboxylic acid (TCA) cycle, such as isocitrate dehydrogenase, malate dehydrogenase (Kilgour, 1980), and α-oxoglutarate oxidase (Flynn and Bowman, 1973). Glucose is now converted to succinate, acetate and CO_2, which are the main end products excreted. The oxidative metabolism of amino acids (especially proline) by the functional Krebs cycle and the respiratory chain serves as the major source of energy in this stage of the life cycle. Proline is present at concentrations up to 150mM in the insect where it serves as a source of energy for flight (Bursell, 1966).

Glucose metabolism in the bloodstream form trypanosome differs from glycolysis in other eukaryotes in a number of respects: (a) Lactate dehydrogenase is absent (Dixon, 1966), and therefore the reducing equivalents generated in glycolysis are indirectly reoxidised by molecular oxygen *via* a dihydroxyacetone phosphate (DHAP):glycerol 3-phosphate (G-3-P) shuttle plus a cyanide-insensitive mitochondrial oxidase: G-3-P oxidase.
(b) Under aerobic conditions, pyruvate is the sole end product of glycolysis and is excreted into the host's bloodstream. (c) Under anaerobic conditions glucose is quantitatively converted into equimolar amounts of pyruvate and glycerol, which are excreted (Fairlamb *et al.* , 1977) and for every molecule of glucose consumed, one ATP molecule is produced. A similar situation arises under aerobic conditions in the presence of the metal-chelating agent salicylhydroxamic acid (SHAM), which acts as an inhibitor of α-glycerophosphate oxidase, producing an effect which stimulates anaerobiosis (Evans and Brown, 1973; Opperdoes *et al.* , 1976b) . It is the coupling of the Embden-Meyerhof pathway to the unique L-glycerol-3-phosphate oxidase system (Grant and Sargent, 1960) which maintains oxidation-reduction balance and adequately accounts for the carbon flow and energy balance under aerobic conditions (Opperdoes *et al.* , 1976a).

Under anaerobic conditions equimolar amounts of pyruvate and glycerol are produced (Grant and Fulton, 1957; Brohn and Clarkson, 1980) by a

mechanism which is generally accepted to follow the Embden-Meyerhof pathway although reservations have been expressed as to its limitations (Ryley, 1962; Opperdoes *et al.* , 1976b). The compartmentalisation of a number of glycolytic enzymes in the glycosome has been used to explain the anaerobic pathway converting glucose to equimolar pyruvate and glycerol while giving a net yield of ATP (Opperdoes and Borst, 1977) . Hammond and Bowman (1980) have shown that glycerol formation may proceed within the glycosomal compartment of the trypanosome cell *via* reversal of the glycerol kinase reaction resulting in ATP synthesis by L-glycerol-3-phosphate-dependent phosphorylation of ADP. This thermodynamically unfavourable reaction may proceed due to efficient compartmentation of the trypanosome cell leading to high local concentrations of G-3-P. This factor coupled with glucose phosphorylation from G-3-P-dependent ATP formation would allow this reaction to proceed in the direction of ATP generation (Kohler, 1982).

^{13}C nuclear magnetic resonance (NMR) studies of anaerobic glycolysis in *T.brucei* spp. have confirmed that the end-products of glucose metabolism were glycerol and pyruvate together with alanine and dihydroxypropionate (Mackenzie *et al.* , 1983). In agreement with Grant and Fulton (1957), the ^{13}C NMR data obtained by Mackenzie *et al.* (1983) showed that C-1 of [1-^{13}C]-glucose is incorporated into the C-1 (= C-3) of glycerol to the extent of 2 to 1 over C-3 of pyruvate. However, in contrast to the early studies, the same incorporation (66 %) of C-6 of [6-^{13}C]glucose into C-3 of pyruvate, alanine, and 2,2-dihydroxypropionate (collectively) over C-1,3 of glycerol was found (Mackenzie *et al.*, 1983). Comparison of whole-cell and acid-lysate high resolution ^{13}C NMR spectra concluded that pyruvate may be initially formed bound to a macromolecular species and that 10% of pyruvate produced was transaminated to alanine *in vivo*. For Scheme 1 in figure 1.7 (Mackenzie *et al.*, 1982) to hold, the rates of formation of glycerol from DHAP and pyruvate from GAP would need to be equal and greater that the rate of the triose phosphate isomerase-mediated conversion of GAP into DHAP. The indication of



SCHEME 1

Figure 1.7. Embden-Meyerhof glycolytic pathway showing the fate of C-1 (•) and C-6 (✳) of glucose in *T.b.brucei* bloodstream forms. Equilibrium (a) denotes the triosephosphate isomerase reaction. P in a circle represents phosphate. Abbreviations: G-6-P, glucose-6-phosphate ; FDP, fructose-1,6-diphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; G-3-P, L-glycerol-3-phosphate; 1,3-DPGA, 1,3-diphosphateglycerate (Mackenzie *et al.*, 1982).

Mackenzie *et al.* (1983), that there is rapid equilibration in the triose phosphate isomerase reaction, adds to the discrepancy between the results obtained by the different workers.

These observations together with the equimolar production of glycerol and pyruvate provide compelling evidence that Scheme 1 (figure 1.7) does not give a true picture of anaerobic glycolysis and that an alternative pathway must exist.

These observations cannot be reconciled by the high degree of compartmentation in *T.brucei* spp. as no transmembrane processes were involved. This was due to the inhibition of the promitochondrial G-3-P-oxidase under anaerobic conditions which caused the concentration of cytosolic DHAP to rapidly diminish and inactivated the putative specific translocator for DHAP in the DHAP-G-3-P shuttle (Visser *et al.* , 1981). Some of the glycolytic enzymes associated with glycosomes, for example, D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are present also in the cytosol, where they are represented by a different isoenzyme (Opperdoes *et al.* , 1986; Misset *et al.* , 1987).

One theoretical alternative involves the transphosphorylation between glycerolphosphate and a hexose monophosphate, avoiding the loss of one phosphate per glucose in Scheme 1 (figure 1.7) , when glycerolphosphate is hydrolysed to glycerol and inorganic phosphate. Another alternative involves the splitting of a hexosemonophosphate into a triosephosphate and a triose. This would create a hydrogen acceptor (the triose) to re-oxidise NADH without the investment of a phosphate. Both pathways would allow glycolysis to operate anaerobically with a yield of one phosphate per glucose, rather than the 2 phosphates per glucose obtained aerobically. However, the enzymes catalysing such reactions have not been reported in trypanosomes.

Glucose has to pass the plasma membrane, the cell-sap and the glycosomal membrane before it can be phosphorylated by hexokinase. Gruenberg *et al.* (1978), suggested that the entry of D-glucose into the cell might

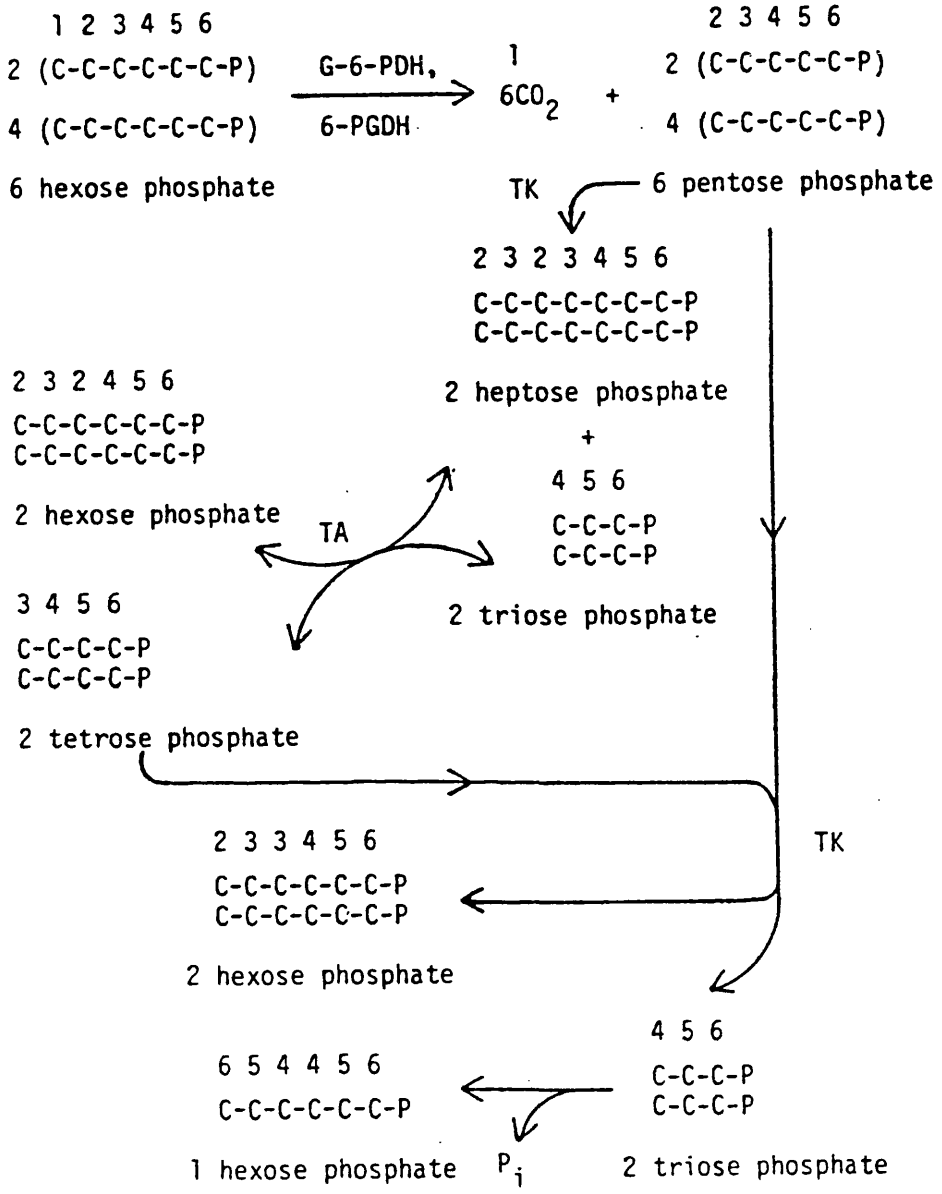
be the rate limiting step in glycolysis, and Nwagwu and Opperdoes (1982) came to the same conclusion in their studies of the regulation of glycolysis in *T.brucei*. Comparison of the kinetic constants of D-glucose metabolism with those for 6-deoxy-D-glucose transport, by Eisenthal *et al.* (1989), has shown that transport across the plasma membrane was likely to be the rate-limiting step of glucose utilisation. The very high transport rate of glucose into the cell is required to match the high metabolic capacity of these organisms.

1.9. The pentose phosphate pathway of glucose metabolism.

It is generally recognised that the pentose phosphate pathway (PPP) fulfills two main functions in metabolism, the formation of ribose 5-phosphate (R5P) for nucleotide and nucleic acid synthesis and the generation of NADPH as a source of reducing equivalents for biosynthetic reactions. In addition, other intermediates of the pathway such as erythrose 4-phosphate or sedoheptulose 7-phosphate may be needed by certain microorganisms for the manufacture of aromatic amino acids or the incorporation into cell wall glycolipids respectively (Srinivasan *et al.*, 1955; Eiderls and Osborn, 1971).

The pathway was formulated in its classical form as a cycle by Horecker and Mehler (1955). The sequence of reactions is shown in Scheme 2 (figure 1.8) in which six molecules of hexose phosphate traverse the cycle with the formation of six molecules of carbon dioxide and six molecules of pentose phosphate. These six molecules of pentose phosphate are converted into four molecules of hexose phosphate and two molecules of triose phosphate. The two triose phosphate molecules may be condensed together by aldolase to form hexose bisphosphate, which can be split by a phosphatase, forming another molecule of hexose monophosphate and a molecule of inorganic phosphate. Thus, the six molecules of hexose phosphate entering the cycle emerge as five molecules of carbon dioxide derived exclusively from C-1 of glucose 6-phosphate.

Although originally presented as the 'pentose phosphate cycle', it has gradually become clear that the 'cycle' can operate as two separate pathways. The



SCHEME 2

Figure 1.8. Distribution of carbon atoms after passage of six molecules of hexose phosphate through the classical pentose phosphate pathway (Axelrod, 1967).

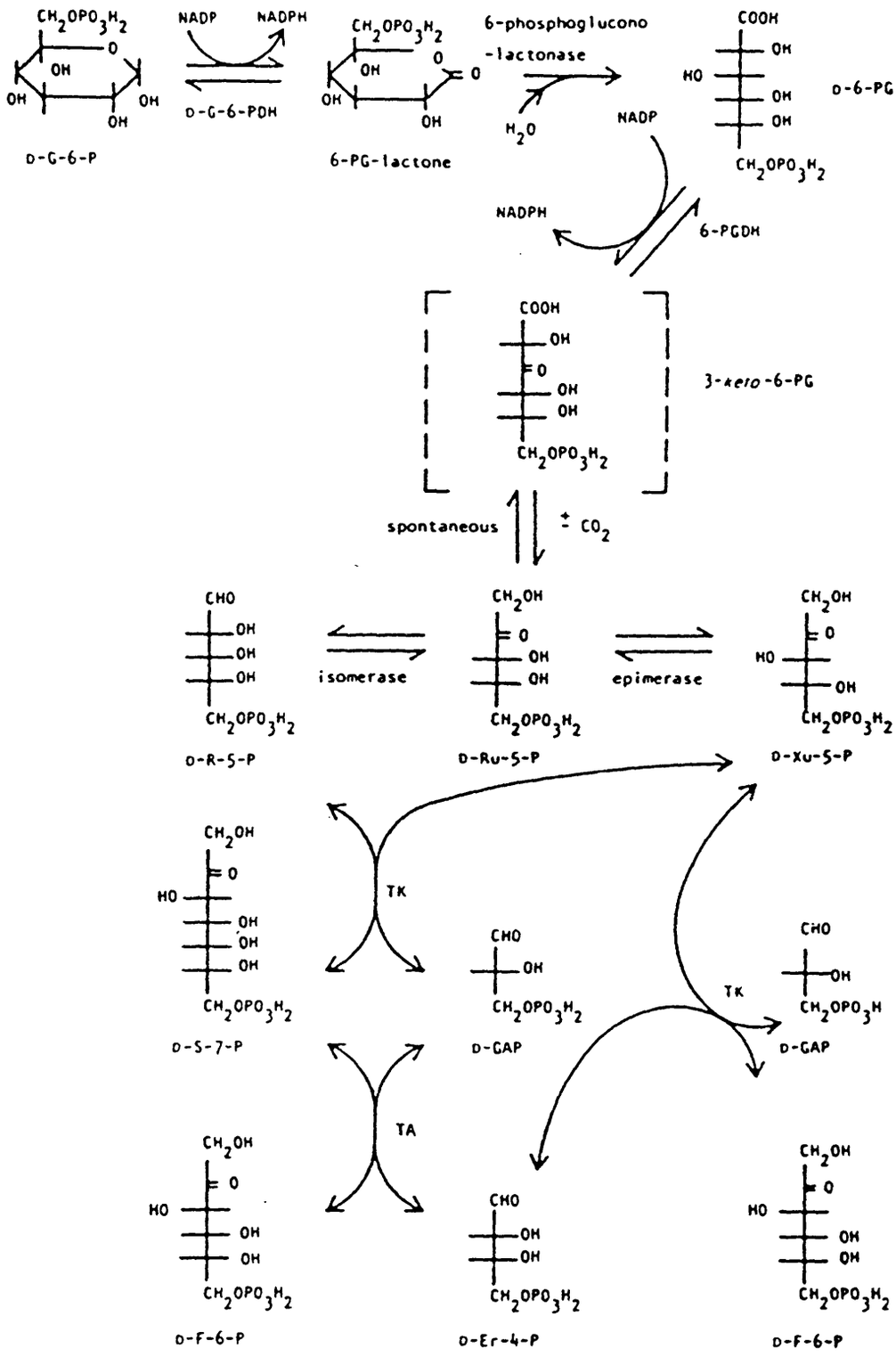
'oxidative branch' generates pentose phosphate and NADPH *via* the two dehydrogenases at the start of the pathway. The non-oxidative branch equilibrates the pentose phosphates with the hexose phosphates of glycolysis *via* the remaining enzymes of the pathway, in particular transketolase (TK) and transaldolase (TA) (Spydevold *et al.*, 1978). Pentose phosphate formed by the oxidative branch and not further utilised is converted back to hexose phosphate by the non-oxidative branch and re-enters the glycolysis pathway.

The 'classical' pathway, as formulated by Horecker and Mehler (1955) leads to the redistribution of the carbon atoms of hexose phosphate illustrated in scheme 3 (figure 1.9; Axelrod, 1967)

1.10. Pentose phosphate pathway in *T.brucei.*

As early as 1957, Grant and Fulton showed that bloodstream forms of the African trypanosomes metabolised isotopically labelled glucose by a classical glycolytic pathway. The pentose phosphate pathway in which the C-1 of glucose is evolved as CO_2 did not appear to be significant under the conditions used, since only 0.6% of [1-^{14}C]glucose and 0.7% of [3,4-^{14}C]glucose was evolved as $^{14}CO_2$. This is in contrast to *T.cruzi*, where there is a marked differential release of $^{14}CO_2$ from [1-^{14}C]glucose and [6-^{14}C]glucose of 28% and 5.2%, respectively (Bowman *et al.*, 1963).

Ryley (1962), demonstrated the presence of low amounts of glucose 6-phosphate dehydrogenase activity in both cultured procyclic and bloodstream forms of *T.b.rhodesiense*. The significance of glucose 6-phosphate dehydrogenase was obscure in the apparent absence of the second enzyme 6-phosphogluconate dehydrogenase (Ryley, 1962). The absence of Mg^{2+} in the assay may have been a possible reason for the lack of detection of this enzyme, since recent screening of bloodstream and cultured procyclic forms of *T.brucei* has shown that they contained glucose 6-phosphate dehydrogenase, 6-phosphogluconolactonase, 6-phosphogluconate dehydrogenase, ribose 5-phosphate isomerase and transaldolase. Only procyclic forms had detectable



SCHEME 3

Figure 1.9. The classical formulation of the pentose phosphate pathway (Horecker and Mehler, 1955).

activities (>0.5 nmole.min^{-1}.mg cell protein^{-1}) of ribulose 5-phosphate 3'-epimerase and transketolase (Cronin *et al.*, 1989).

It seems probable that only the procyclic forms are capable of relying on the non-oxidative branch of the classical PPP to cycle carbon between pentose and hexose phosphates in order to produce glyceraldehyde 3-phosphate as a net product of the pathway. Both forms lack the key gluconeogenic enzyme, fructose-bisphosphatase. Consequently, procyclic forms are incapable of returning any of the glyceraldehyde 3-phosphate produced in the PPP to glucose 6-phosphate, and neither form is able to engage in gluconeogenesis. In addition, neither form had detectable activities of the key enzymes of the Entner-Doudoroff pathway, 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase (Ryley, 1962; Cronin *et al.*, 1989).

The results of Cronin *et al.* (1989) clearly demonstrated that both cultured procyclic and bloodstream forms of *T.brucei* contain the enzymic capacity to metabolise glucose *via* the oxidative segment of the classical PPP in order to produce R5P for the synthesis of nucleic acids and reduced NADP for other synthetic reactions.

The importance of the PPP in the production of most of the NADPH used for biosynthetic processes is not relevant to parasites. Trypanosomes get their NADPH by the action of malic enzyme, so the only essential role of the pathway is to produce pentose for nucleic acid synthesis as in HeLa cells (Reitzer *et al.*, 1980). The cellular growth rate of HeLa cells was proportional to the rate of pentose phosphate synthesis. In rat adipose tissue both the PPP and malic enzyme provide the NADPH requirement of these cells (Gumaa *et al.*, 1973).

1.11. Alternative routes for ribose-5-phosphate synthesis.

In addition to the PPP formation of R5P, other routes also exist by which ribose phosphates are formed:

(a) Direct phosphorylation of pentoses.

Direct phosphorylation of pentoses may lead either to ribose-5-phosphate, or to other pentose-5-phosphates from which it can be derived. Three such reactions are listed below:

Ribose + ATP ------> Ribose-5-P + ADP.

Ribulose + ATP ------> Ribulose-5-P + ADP.

Xylulose + ATP ------> Xylulose-5-P + ADP.

All three enzymes have been reported in various microorganisms, and low activities have also been found in animal tissues such as liver and thyroid.

(b) Phosphoribomutase reaction.

Ribose-5-phosphate can be formed from ribose-1-phosphate by the action of the enzyme phosphoribomutase:

Ribose-1-P <------> Ribose-5-phosphate.

This reaction can also be catalysed by phosphoglucomutase and has been studied by Klenow and Emberland (1955).

Ribose-1-P can also originate from the phosphorolysis of nucleosides through the action of nucleoside phosphorylases, as shown below:

Nucleoside + Pi <------> Ribose-1-P + Base.

Mg^{2+}

1.12. Carbohydrate metabolism in *T.cruzi*.

The substrates utilised and the catabolic pathways present in the mammalian forms of *T.cruzi* are similar to those of culture forms of the organism and are quite distinct from those of the bloodstream forms of the African trypanosomes (Rogerson and Gutteridge, 1979). Culture, blood and intracellular forms of *T.cruzi* have a high rate of endogeneous oxygen uptake and probably

utilise amino acids and carbohydrates as their exogenous energy sources (Rogerson and Gutteridge, 1980).

The culture epimastigote forms can survive incubation in Krebs-Ringer solution without added glucose for 24 hours. The rate of respiration decreases by about 40% of its initial rate but motility is maintained. About 6μmol of oxygen are utilised by 10^8 epimastigotes over a 24 hour period, equivalent to about 5% of the dry weight of the organisms (Rogerson and Gutteridge, 1980). During starvation triglyceride reserves are broken down to free fatty acids and a certain amount of protein degradation also takes place (Oliveria *et al.*, 1977).

Glycolysis functions in all forms as the levels of hexokinase, phosphofructokinase and pyruvate kinase are high. Both epimastigote and trypomastigote forms of *T.cruzi* further metabolise pyruvate to acetate, succinate and carbon dioxide and their mitochondria contain a full complement of cytochromes and TCA cycle enzymes (Gutteridge and Rogerson, 1979; Marr, 1980).

The two primary enzymes in the pentose phosphate pathway, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase have been demonstrated (Raw, 1959; Cannata and Cazzulo, 1984) in *T.cruzi* cells. Lack of conversion of R5P into lactate by cell homogenates of *T.cruzi* epimastigotes led Raw (1959) to the conclusion that the shunt pathway was inoperative. Bowman *et al.* (1963) and Mancilla and Naquira (1964) investigated $^{14}CO_2$ and ^{14}C-glycerol formation from [2-^{14}C]-, [6-^{14}C]- and [U-^{14}C]-glucose by whole cells and showed that the pentose phosphate pathway contributed 28% and 42% towards glucose catabolism in the Peruvian and Tulahuen strains respectively (Mancilla and Naquira, 1964). Terminal catabolism of glucose undoubtedly occurs *via* the tricarboxylic acid cycle, but as with the pentose phosphate shunt, the quantitative contribution of the cycle appears to differ between different strains of *T.cruzi* (Mancilla and Naquira, 1964).

1.13. 5-Phosphoribosyl-α-1-pyrophosphate (PRPP).

The discovery of PRPP took place in 1955 in two independent laboratories, by Kornberg, Lieberman, and Simms in St. Louis, Missouri, and by Remy, Remy, and Buchanan in Cambridge, Massachusetts. The structure of PRPP is shown in figure 1.10(a).

PRPP is the essential substrate for supply of the ribose moiety in the biosynthesis of purine, pyrimidine, and pyridine nucleotides (Henderson and Paterson, 1973). It occupies a unique position connecting the major metabolic pathways of the pentose phosphate shunt and nucleotide synthesis. In microorganisms and plants, it is also a precursor for tryptophan (Yanofsky, 1956), and histidine biosynthesis. In animal cells , PRPP also participates in the synthesis of ribonucleotides of imidazoleacetic acid (Gots, 1957), quinolic acid (Wyngaarden, *et al.* , 1958), and histamine (Preiss and Handler, 1957). Because of its involvement in such a great number of metabolic pathways, the amount of PRPP in a bacterial cell (the PRPP pool) varies depending on growth conditions (Sadler and Switzer, 1977).

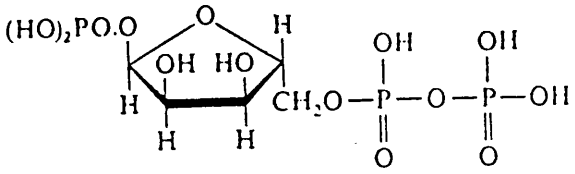
PRPP is formed from ribose-5-phosphate (R5P) and adenosine-5-triphosphate (ATP) by PRPP synthetase (ATP : D-ribose-5-phosphate pyrophosphotransferase, EC 2.7.6.1) according to the following equation:

$$R5P + ATP \xleftarrow{Mg^{2+},Pi} AMP + PRPP$$

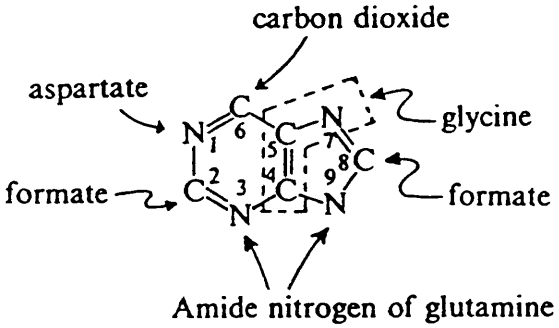
The equilibrium constant (Keq) for the PRPP synthetase reaction was estimated by Switzer (1969), and found to have an average value of 28.6 at pH 7.5, 5 mM $MgCl_2$ and 37° C.

As PRPP is an essential precursor for a number of divergent metabolic pathways it is likely that synthesis of this compound is tightly controlled by feedback mechanisms. Switzer (1967) and Atkinson and Fall (1967) have indicated that feedback inhibition of bacterial PRPP synthetase does occur and the latter workers have shown that the activity is regulated by the relative concentrations of AMP, ADP, and ATP (see also Klungsoyr *et al.* , 1968). The

(a)



(b)



The Basic Precursors of the Purine Skeleton.

(c)

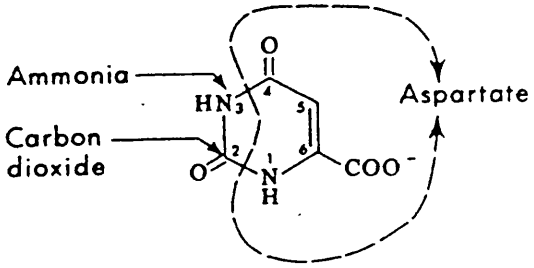


Figure 1.10. (a) Structure of PRPP.

(b) The basic precursors of the purine skeleton.

(c) The basic precursors of the orotate ring.

enzymatic activity is modulated by many effectors: Mg^{2+} and inorganic phosphate as activators; and ADP, 2,3-bisphosphoglycerate , or GDP as competitive or noncompetitive inhibitors (Becker *et al.* , 1979).

From a mechanistic viewpoint, the enzyme is interesting because it bridges the gap between other nucleotide-utilising enzymes. Kinases are involved in mechanisms where nucleophilic attack occurs at the γ-phosphorus and nucleotidyl transferases are involved in mechanisms where nucleophilic attack occurs at the α-phosphorus of ATP. PRPP synthetase is a pyrophospho-kinase; nucleophilic attack occurs at the β-phosphorus, and an intact pyrophosphoryl group is transferred from ATP to R5P (Miller *et al.* , 1975). The reaction is one of two such known. The second pyrophosphokinase known catalyses the conversion of thiamine to thiamine pyrophosphate (Snyder and Speck, 1939).

1.14. The *de novo* pathway of purine nucleotide biosynthesis.

Purines are formed *in vivo* by the synthesis of first the imidazole ring then the pyrimidine ring. Using partially purified enzymes from pigeon liver, Buchanan and his co-workers were able to unravel the steps in the enzymic synthesis of inosinic acid, the first product formed with a complete purine ring structure (Buchanan, 1961). The reactions involved in purine *de novo* synthesis are shown in figure 1.11.

The basic precursors of the purine skeleton are shown in figure 1.10(b). The ability to synthesise the purine ring system *de novo* is by no means a universal biochemical capacity. All the protozoan parasites studied thus far appear to be unable to synthesise purines *de novo* (Wang, 1982). Trypanosomes resemble those mammalian cells, for example, heart muscle (Liu and Feinberg, 1971), platelets (Sixma *et al.* , 1976) and mature erythrocytes (Gerlach *et al.* , 1965), which cannot synthesise purines *de novo* and possess high affinity uptake systems for adenosine. Purine metabolism studies in bloodstream forms of African trypanosomes have shown that purine bases label the nucleotide pools

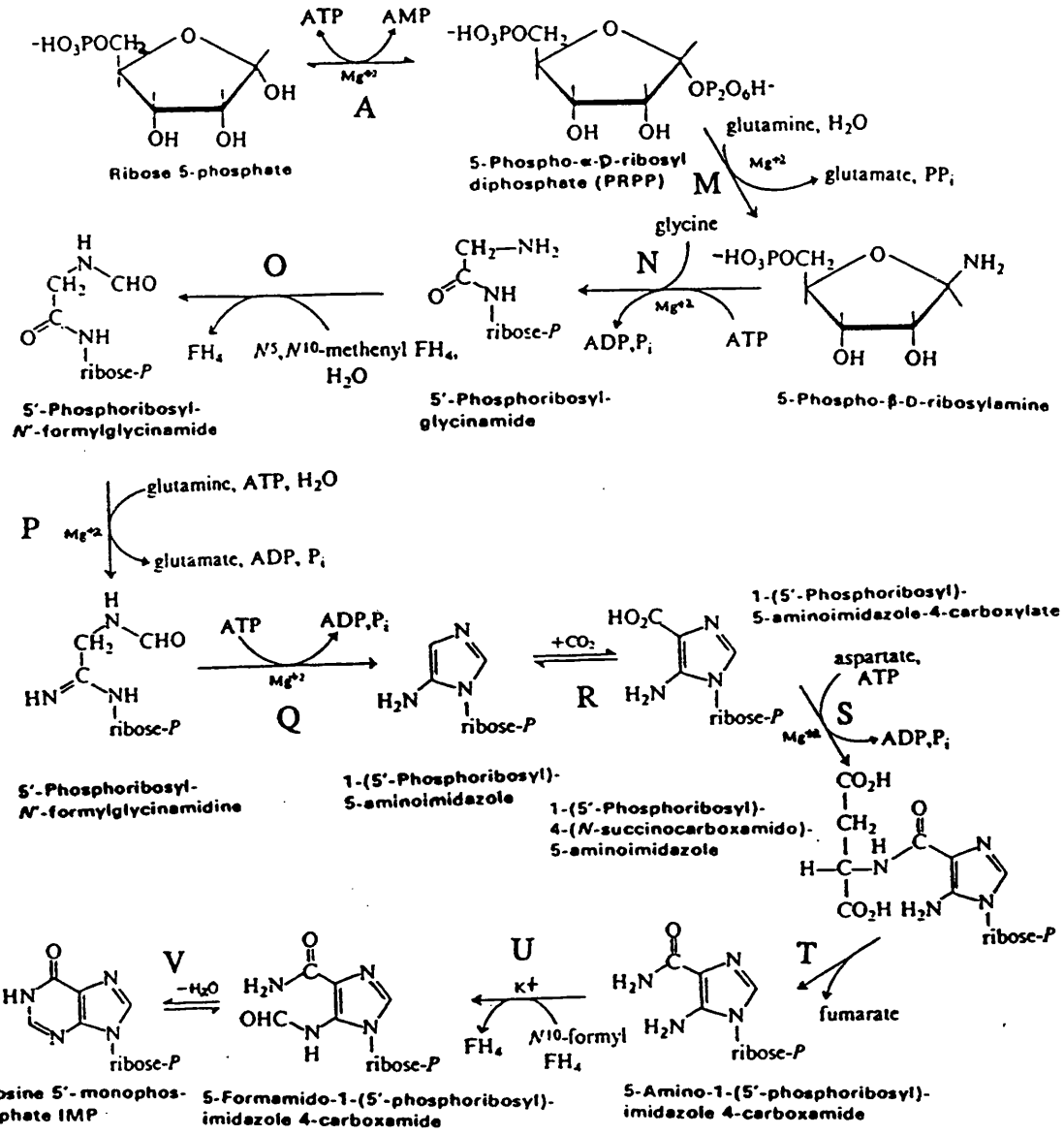


Figure 1.11. Enzymes involved in the biosynthesis of Inosine 5'-monophosphate (Mahler and Cordes, 1968).

Enzyme activities: A, ribose-5-phosphate pyrophosphokinase; M, phosphoribosyl-diphosphate amidotransferase; N, phosphoribosyl-glycinamide synthetase; O, phosphoribosyl-glycinamide formyltransferase; P, phosphoribosyl-formylglycinamide synthetase; Q, phosphoribosyl-aminoimidazole synthetase; R, phosphoribosyl-aminoimidazole carboxylase; S, phosphoribosyl-succinocarboxamido-aminoimidazole synthetase; T, adenylosuccinate lyase; U, phosphoribosyl-aminoimidazole-carboxamide formyltransferase; V, IMP cyclohydrolase.

more efficiently than ribonucleosides (Fish *et al.*, 1982b). Although the last two enzymes of the *de novo* pathway are known to be present in the mature mammalian erythrocytes, PRPP amidotransferase, the first enzyme, appears to be missing (Fontenelle and Henderson, 1969).

Fish *et al.* (1982a), incubated bloodstream forms of *T.b.gambiense* and *T.b.rhodesiense* with [U-^{14}C] radiolabelled formate, glycine or serine and found no detectable incorporation of radiolabel into any of the purine ribonucleotides. This suggested the absence of any *de novo* purine synthesis. *De novo* purine synthesis does not occur in procyclic forms of *T.b.gambiense* (Fish *et al.*, 1982b), as well as in none of the major morphological forms of *T.cruzi*, the amastigote, trypomastigotes and epimastigotes (Gutteridge *et al.*, 1979).

1.15. The *de novo* pathway of pyrimidine nucleotide biosynthesis.

A central distinction between the metabolic routes leading to the formation of purine and pyrimidine nucleotides is the timing of the formation of the N-glycosidic bond. In contrast to the purine biosynthetic pathway, the complete pyrimidine nucleus is synthesised prior to its attachment to R5P; the key intermediate introduced into the N-glycosidic linkage is orotic acid, uracil-4 carboxylic acid, which contains the pyrimidine nucleus. Dihydro-orotic acid is formed by the cyclisation of the reaction product of aspartic acid and carbamyl phosphate (Crosbie, 1960). Glutamine, ATP, HCO_3^- and aspartate were recognised as the precursors of particulate atoms of the orotate ring (Reichard and Lagerkvist, 1953), as shown in figure 1.10(c).

There are 6 enzymes involved in the pyrimidine *de novo* nucleotide biosynthetic pathway which is summarised in figure 1.12. All 6 enzymes of pyrimidine biosynthesis *de novo* have been detected in homogenates of the culture promastigote form of *L.m.amazonensis*, the blood trypomastigote form of *T.brucei* and the culture epimastigote, blood trypomastigote and intracellular amastigote forms of *T.cruzi* (Gutteridge and Gaborak, 1979).

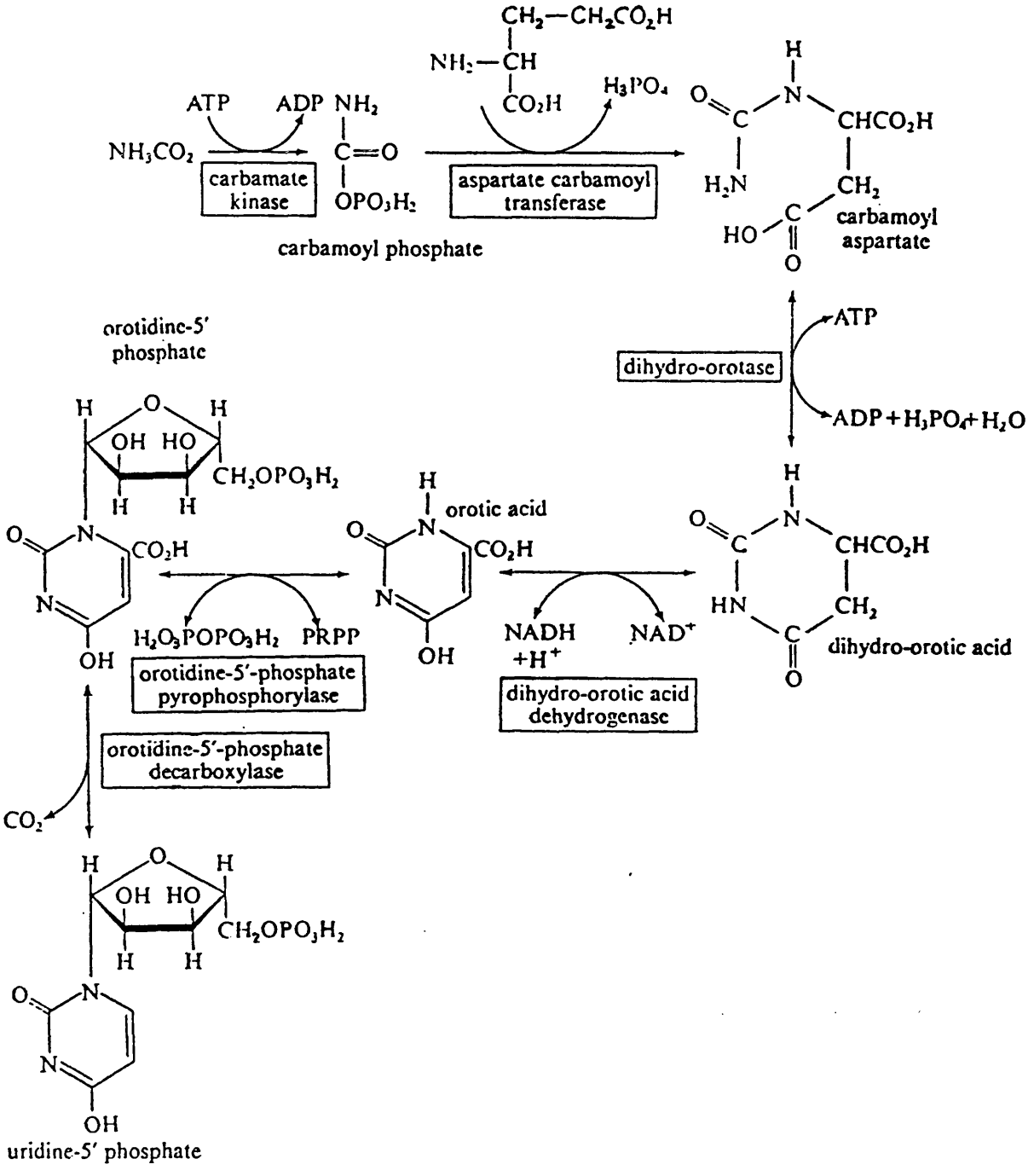


Figure 1.12. Reactions involved in the synthesis of Uridine-5'-Phosphate (Cohen, 1965).

The subcellular distribution of pyrimidine biosynthetic enzymes in members of the Kinetoplastida is different to that described for other species studied to date (Hammond *et al.*, 1981). The intracellular location for the enzymes of UMP synthesis in (a) Kinetoplastida and (b) mammals is shown diagramatically in figure 1.13 (Hammond and Gutteridge, 1982).

1.16. Salvage pathways for purine and pyrimidines.

Parasites rely on their environment to provide a source of purines. In the absence of *de novo* synthesis, it is not surprising to find elaborate systems for absorption and interconversion of existing purines. The latter are the so-called 'salvage' pathways, widely distributed in parasites.

Gutteridge and Davies, (1981), detected substantial incorporation of radiolabelled purine bases and nucleosides but not nucleotides into nucleic acids. Cell-free homogenates of all three main forms of *T.cruzi* contained a range of phosphoribosyltransferases, aminohydrolases, kinases, hydrolases and phosphorylases which are involved in the salvage mechanism of purines. The properties of these purine phosphoribosyltransferases were studied by Gutteridge and Davies (1981, 1982) who showed that phosphoribosyltransferase was the sole activity involved in hypoxanthine salvage and also played a major role in adenine and guanine salvage. They also demonstrated that in the culture epimastigote forms of *T.cruzi* purine phosphoribosyltransferase activity involves two separate enzyme proteins with different subcellular distribution. One, active with adenine as substrate and is found in the cytosol; the other, active with both hypoxanthine and guanine, occurs in the glycosomes.

It has been shown by James and Born (1980), that bloodstream forms of *T.b.brucei* can take up various nucleosides and bases with different apparent order of salvage efficiency to *T.b.gambiense* and *T.b.rhodesiense* (Fish *et al.*, 1982a,b). The order of salvage efficiency for purine bases and their respective nucleosides in *T.b.gambiense* and *T.b.rhodesiense* is: adenine>hypoxanthine>

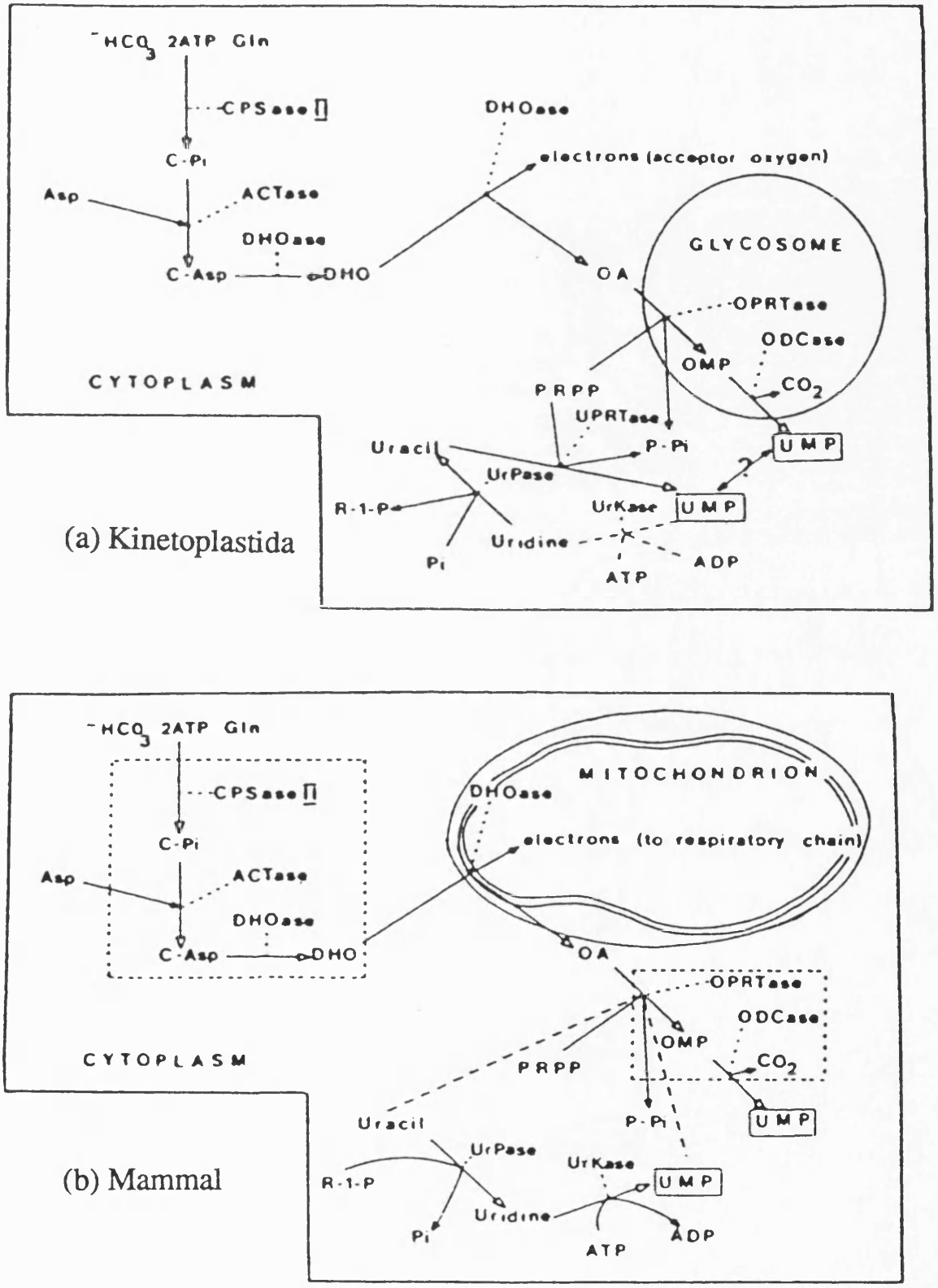


Figure 1.13. Diagram of the intracellular location for the enzymes of UMP synthesis in (a) Kinetoplastida and (b) mammals (Hammond and Gutteridge, 1982).

guanine>xanthine, with the order of purine bases of adenine and hypoxanthine reversed in *T.b.rhodesiense* (Fish *et al.*, 1982a,b). Although the incorporation of labelled purine bases into the nucleotide pool is generally more efficient than that of ribonucleosides, the rate of uptake of adenosine in bloodstream forms of *T.b.brucei* is much greater than that of the other purines and purine nucleosides, the uptake of which decreased in the order adenine, inosine, guanosine and hypoxanthine (James and Born, 1980). The concentration of adenosine is very low (10^{-8}-10^{-7} M) in normal plasma (Miyazaki *et al.* , 1974). However, there is evidence of a rapid turnover of adenosine in the nucleotides of erythrocytes in normal blood (Adams and Harkness, 1973; Henderson and LePage, 1959; Mager *et al.* , 1967) so that a source normally accessible to red cells could presumably also be available to trypanosomes in the blood; there is evidence that purines, mainly nucleosides, are continuously released from various tissues including myocardium (Rubio and Berne, 1969). It is probable also that the destructive effect of trypanosomal infections on the tissues increases the concentration of adenosine and other purines in the extracellular fluids where they can be utilised by the parasites (James and Born, 1980).

Phosphoribosyltransferases catalyse the formation of purine and pyrimidine nucleotides from the free base and PRPP as follows:

Base + PRPP <------> base nucleotide + PPi

Nucleoside phosphorylases catalyse the formation of nucleosides from the bases and R1P as follows:

Base + R1P <------> base nucleoside + Pi

The nucleosides are converted to nucleotides through the action of an appropriate kinase:

Ribonucleoside + ATP -----> nucleotide + ADP

Two successive kinase reactions convert mononucleotides into the actual precursors of RNA, the nucleoside triphosphates:

$$\text{NMP} \xrightarrow[Mg^{2+}]{ATP\ ADP} \text{NDP} \xrightarrow[Mg^{2+}]{ATP\ ADP} \text{NTP}$$

These transphosphorylation reactions are readily reversible since the breakage of each phospho-anhydride bond in the substrate results in the formation of another such bond in the product.

The biochemical transformations converting inosine 5-phosphate (inosinic acid) to adenosine 5-phosphate (adenylic acid) and guanosine 5-phosphate (guanylic acid) are indicated in figure 1.14.

Figure 1.15 summarises the enzymes involved in purine salvage in *T.cruzi*, *T.brucei*, and *L.mexicana* (Davies *et al.* , 1983; Fish *et al.* , 1982a).

Hypoxanthine-guanine phosphoribosyltransferase (HGPRTase) plays an important part in the reutilisation of purines by liver and other tissues (Pritchard *et al.* , 1970). In man, HGPRTase deficiency leads to Lesch-Nyhan syndrome, a rare inherited disorder that is characterised by overproduction of uric acid and neurologic abnormalities including a compulsive form of self-mutilation (William and Wyngaarden, 1983).

As regards pyrimidines, Trypanosoma spp. are reliant equally on salvage and on *de novo* synthesis. All enzymes of UMP production *via* the salvage pathway are soluble. Uridine salvage in the Kinetoplastida involves phosphorolysis to form uracil, catalysed by uridine phosphorylase and not by uridine kinase. Uridine kinase has not been detected in any of the Kinetoplastida, yet is detectable in rat liver (Hammond and Gutteridge , 1982) . It is quite likely therefore that uridine salvage involves phosphorolysis to form uracil, catalysed by uridine phosphorylase, which may then be converted to UMP by uracil phosphoribosyltransferase (Jaffe, 1961). The lack of detection of both uracil phosphoribosyltransferase and uridine kinase in the amastigote form of *T.cruzi* suggests that this form has a more limited ability to salvage uracil and uridine

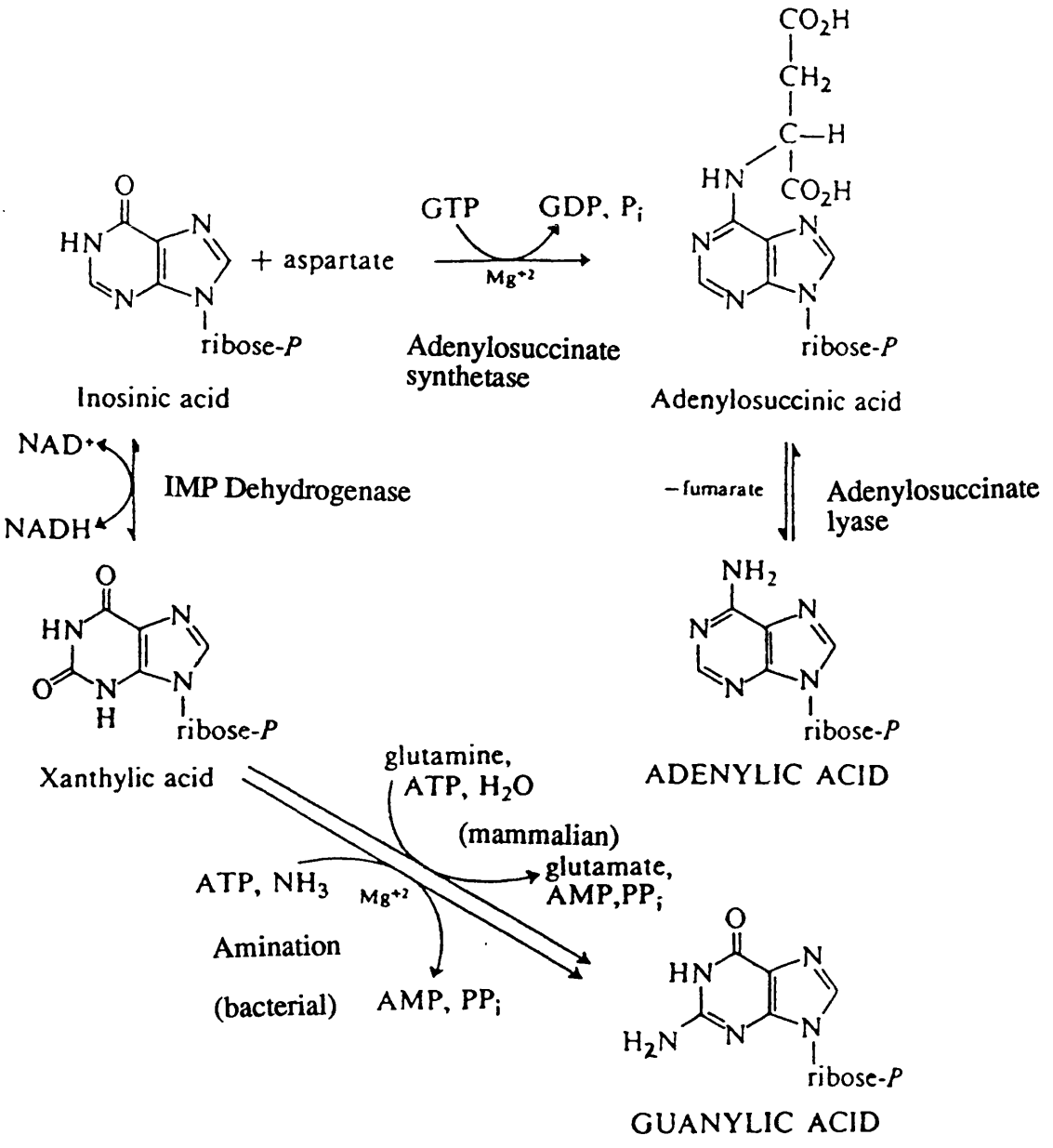


Figure 1.14. The conversion of Inosinic acid to Adenylic and Guanylic acids (Cohen, 1965).

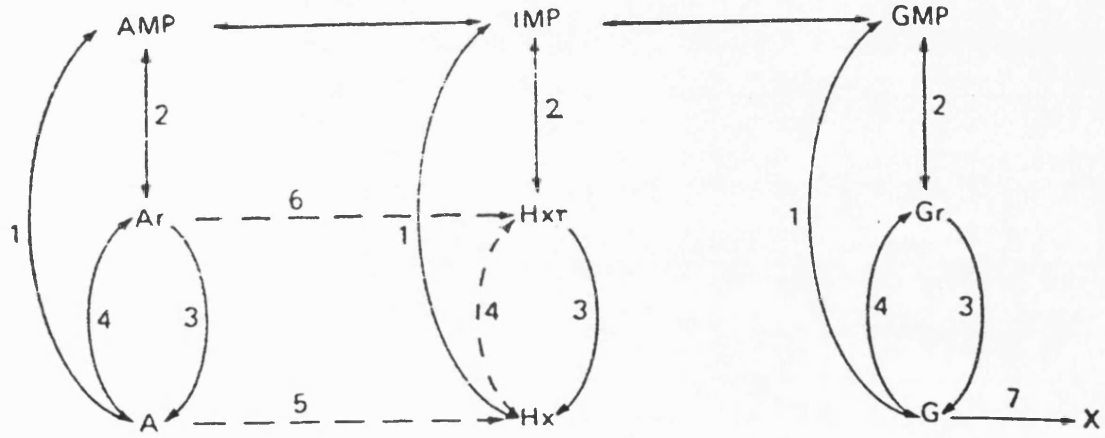


Figure 1.15. Enzyme activities involved in purine salvage pathways of the Trypanosomatidae (Henderson and Paterson, 1973).

Enzyme activities: (1) phosphoribosyltransferase; (2) nucleoside kinase; (3) nucleoside hydrolase; (4) nucleoside phosphorylase; (5) adenine aminohydrolase; (6) adenosine aminohydrolase; (7) guanine aminohydrolase. The dotted lines indicate that enzyme is absent in one or more of the organisms.

than the epimastigote and trypomastigote forms (Gutteridge and Gaborak, 1979). In contrast mammalian cells salvage uracil preferentially by first forming uridine by uridine phosphorylase and then converting this to UMP by uridine kinase (Hochstadt, 1974).

Cytosine phosphoribosyltransferase activity in trypanosomes is expressed by uracil phosphoribosyltransferase (Hammond and Gutteridge, 1984). Because nucleosides are converted to nucleotides through kinases, bases through phosphoribosyltransferases and because thymine and uracil could first be converted to nucleosides, cytosine and uracil bases are interconvertable, and uracil alone could supply all the pyrimidine requirements of the cell.

1.17. Control and treatment of African trypanosomiasis.

Currently the most effective method of control of African trypanosomiasis is the avoidance of the tsetse fly by the movement of human populations to tsetse-free areas or reducing tsetse fly-man contact by control of the size and distribution of the population. The methods of vector elimination under investigation include, alterations of the habitat, pesticide application, genetic and biological control, conical fly traps and physiological interference such as interference with insect growth regulators which inhibit chitin synthesis (Molyneux, 1982). Wild game acts as a parasitic reservoir and the measures to conserve these species have made the control of trypanosomiasis a more difficult problem. Several drugs are available (Ethidium, Anthrycide sulphate, Berenil, Samorin) for the treatment of trypanosomiasis in infected animals. Substantial resistance of certain *T.brucei* strains has developed to all of the available drugs except Berenil with serious complications to prophylaxis and treatment - especially of relapse infections (Molyneux and Ashford, 1983).

The incidence of nagana could be reduced by the exploitation of the trypanotolerance of some breeds of cattle. These cattle, primarily humpless taurine breeds, are smaller than the more commonly kept zebu and not such prolific milk-producers. It remains for long-term breeding programmes to combine the most desirable characteristics of the various breeds (Molyneux and Ashford, 1983).

Sleeping sickness due to *T.b.rhodesiense* has been reported in all the following countries of Eastern Africa, Burundi, Ethiopia, Kenya, Malawi, Mozambique, Rwanda, Sudan, Tanzania, Uganda, Zambia and Zimbabwe. *T.b.gambiense* overlaps with the distribution of *T.b.rhodesiense* in the region of the East African complex of great lakes, particularly the northern shores of Lake Victoria in Uganda. Currently the most active foci of *T.b.gambiense* are in Cameroon, Congo, Ivory Coast, Southern Sudan, Uganda and Zaire. In Nigeria extensive vector control activities directed against the animal disease have made an impact on the number of human cases reported, and a vector control

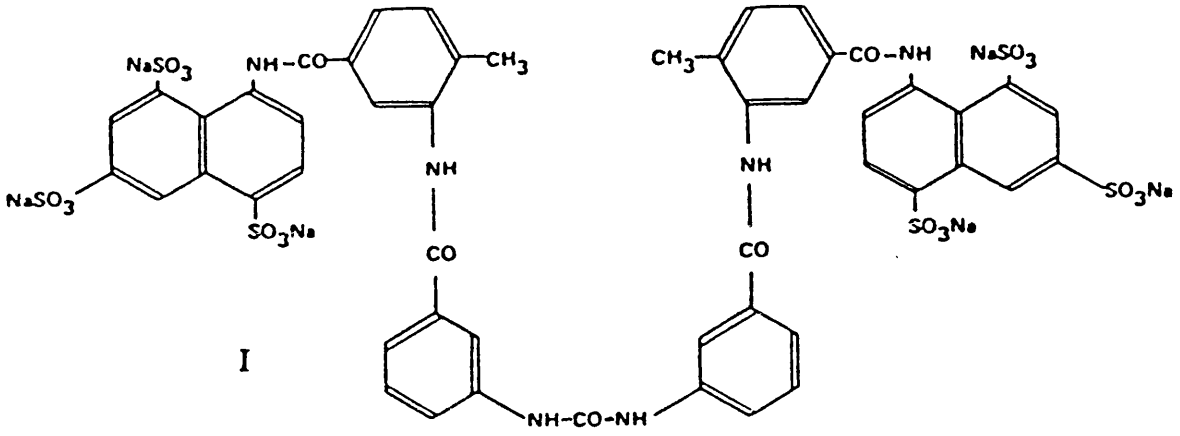
programme combined with treatment has practically eradicated *T.b.gambiense* sleeping sickness in Kenya and Tanzania (Molyneux and Ashford, 1983).

Constant monitoring and rapid treatment of people living in areas in which trypanosomiasis is endemic have prevented serious outbreaks of the disease in recent years. The early stages of sleeping sickness in men are controlled by suramin and occasionally by pentamidine and berenil although the former is used mainly for chemoprophylaxis (see Figure 1.16) . None of these drugs will cross the blood-brain barrier and so control of the late stages of the disease in which invasion of the CNS occurs rests with arsenicals such as tryparsamide and Melarsoprol (MelB, Arsobal) with undesirable side effects. No drug is in routine use to prevent transmission during blood transfusion.

Melarsoprol remains the best available drug for any stage of sleeping sickness. The drug itself causes a mortality of up to 10% due to fatal side effects. It has been found to be highly inhibitory to *T.b.brucei* pyruvate kinase (Bowman and Flynn, 1976; Flynn and Bowman, 1974), but further studies by Van Schaftingen *et al.* (1987) suggest that this may not be so. These workers proposed that the drug blocks the formation of Fru(2,6)P_2 through inhibition of PFK 2. The way by which melarsen oxide causes cell lysis remains to be identified.

The shortness of the treatment period for pentamidine is its main advantage over suramin, where the full course takes weeks. Pentamidine works well in both prophylactic and treatment modes against the Rhodesian disease. It appears to exert its effect on nucleic acid biosynthesis, possibly by binding to DNA by a non-intercalative mechanism due to its positive charge at physiological pH (Newton, 1974). Selectivity appears to be due to differential permeability between host and parasite: trypanosomes contain a pentamidine transport system which raises the intracellular concentration of the drug to many times the plasma concentration (Damper and Patton, 1976).

Suramin, a highly-polar sulphated-naphthylamide is a competitive inhibitor of glycerophosphate oxidase with respect to α-glycerophosphate. The



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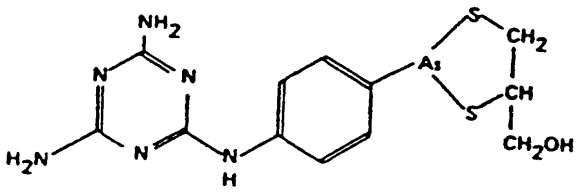

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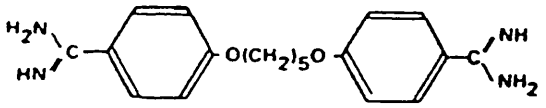

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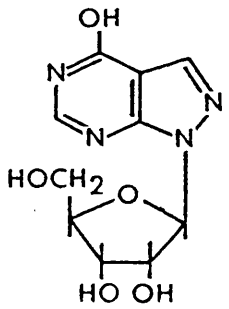

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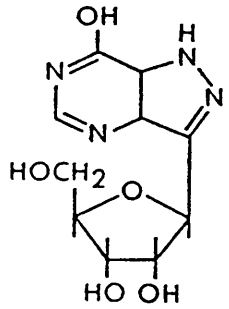


Figure 1.16. Structures of commonly used drugs against African trypanosomiasis.

I = Suramin, II = Melarsoprol, III = Pentamidine,

IV = Allopurinal ribonucleoside, V = Formycin B.

drug is administered intravenously and slowly taken up by the trypanosome by endocytosis in the form of a plasma-protein-bound complex (Gutteridge, 1985). Its effectiveness is limited by its inability to cross the blood-brain barrier (Meshnik, 1984).

Some recent studies of the polyamine metabolism in *T.brucei* have shed some light on the possible rational design of antitrypanosomal agents. α-Difluoromethylornithine, a "suicidal inhibitor" of ornithine decarboxylase, cured *T.b.brucei* infection in mice when administered in drinking water at 0.5% (Bacchi *et al.* , 1980) and was shown to inhibit ornithine decarboxylase in intact *T.b.brucei* cells at a 10 times higher potency than its inhibition of mammalian ornithine decarboxylase. Putrescine, spermine and spermidine can reverse the antitrypanosomal activity of difluoromethylornithine (Nathan *et al.* , 1981). A combination of the drug with the DNA cleaver bleomycin has a synergistic effect on *T.b.brucei*; the combination therapy can cure advanced CNS trypanosomiasis in mice. The rational basis behind this antitrypanosomal activity can probably be explained by the joint interference of rapid DNA replication and cell division in *T.b.brucei.*

In a recent issue of New Scientist (Coghlan, 1990), it was reported that after a long delay of two years, the Food and Drug Administration in the U.S.A. approved difluoromethylornithine, to treat *T.b.gambiense* trypanosomiasis, which is more common in West and Central Africa. It has no effect on *T.b.rhodesiense* infections. The main drawback of the drug is that it must be administered intravenously. This requires medical supervision which increases the cost of treatment and restricts its use to specialised centres rather than in the villages where it is mostly needed. Trials conducted with about 600 patients suffering from advanced trypanosomiasis in Congo and Ivory Coast, have shown that the drug has no serious side effects.

This is one example of the recent progress in rational approaches to antitrypanosomal chemotherapy. Most of the research is being carried out in academic institutions worldwide and the biochemical and molecular biology

expertise currently available render the prospect of new drug development very promising. The aim of these research groups is to discover an enzyme or a unique pathway which exists in the trypanosome and not the host and to inhibit its activity causing a fatal blow to the parasite (Morello, 1988)

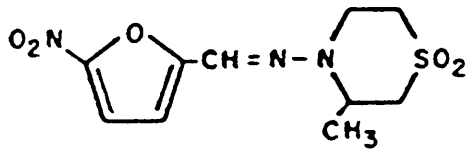
One of the chief obstacles to the design of antitrypanosomal drugs is that although the bloodstream form is free, *i.e.* it is not intracellular and does not occupy an immunologically privileged site, it is able to evade the host's immune system. This is due to the existence of rapidly changing repertoirs of VATs (serodemes). This property has made the prospect of vaccinating against African trypanosomiasis a daunting if not impossible one (Barry, 1989). Therefore, an attempt to develop antitrypanosomal drugs must rely on other biochemical differences between trypanosomes and their mammalian hosts.

1.18. Control and treatment of Chagas' disease.

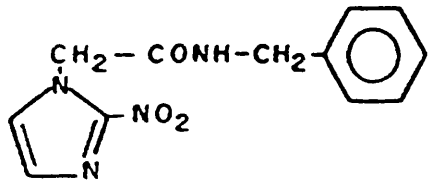
The lack of a registered drug with chemoprophylactic properties is a serious deficiency, especially in areas where there are large potential reservoirs of infection. Any progress in eradication such as vector control and better housing, is often rapidly undone by reinfection from such reservoirs.

Two drugs, a 5-nitrofuran derivative (Nifurtimox, Bayer 2502) and a 2-nitroimidazole, (Benznidazole, Radanil) (see Figure 1.17) are used to treat both acute and chronic phases (Gutteridge, 1985). These drugs have such severe side effects that the full recommended course is rarely completed. The development of more acceptable alternatives has been hampered by the carcinogenic and mutagenic properties of the most promising nitro-compounds (Molyneux and Ashford, 1983).

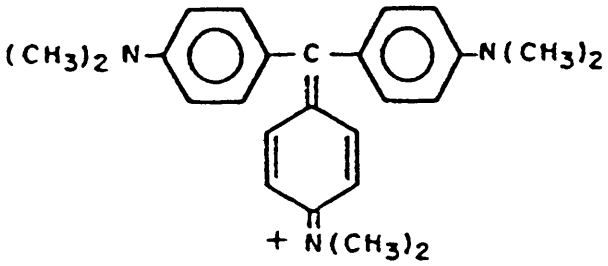
The development of a vaccine against *T.cruzi* has been , until now, unsuccessful. Educational campaigns, especially among young people, are becoming a very useful way to combat the disease (Schofield, 1985). Gentian violet is the chemical currently used to treat transfusion blood. Its main



Nifurtimox



Benznidazole



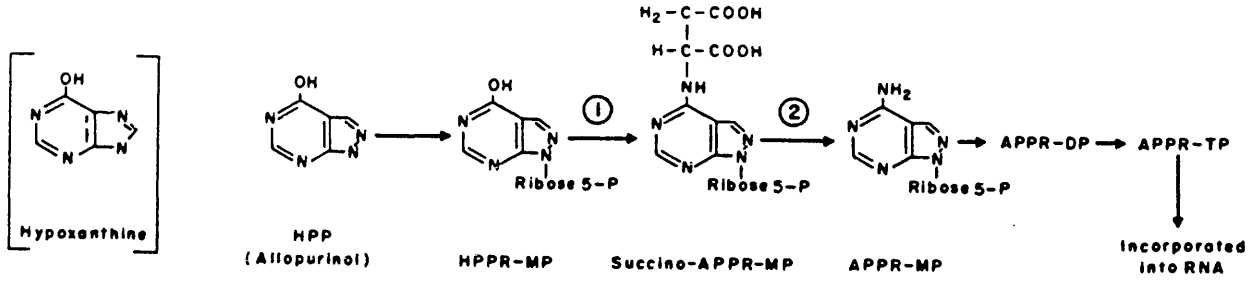
Crystal violet (Gentian Violet)

Figure 1.17. Structures of Nifurtimox, Benznidazole and Crystal Violet (Gentian Violet).

disadvantage is the colouring of blood and the staining of patient's tissues. It has no side effects and is very effective.

Differences in the metabolism of purines between *T.cruzi* and the mammalian host have been utilised for the development of a rational approach to the chemotherapy of Chagas' disease (Marr *et al.*, 1978; Berens *et al.*, 1981). *T.cruzi* cannot synthesise purines *de novo* and depend exclusively on salvage pathways for their purine supply. Some of the purine salvage enzymes appear to have a more relaxed substrate specificity than the host enzymes (Gutteridge and Davies, 1981).

The pyrazolopyrimidine base allopurinol (4-hydroxypyrazolo [3,4-α] pyrimidine, HPP, a structural analogue of hypoxanthine is activated by hypoxanthine phosphoribosyltransferase to the ribonucleotide-5-monophosphate (HPPR-MP). HPPR-MP is aminated to 4-aminopyrazolopyrimidine ribonucleotide (APPR-MP) and subsequently phosphorylated to the triphosphate form and incorporated into RNA inhibiting the parasite's growth *in vitro* (Marr *et al.*, 1978; Avila *et al.*, 1981). The metabolic transformation of allopurinol in *T.cruzi* and human erythrocytes is presented in figure 1.18. Allopurinol is a relatively nontoxic drug which has been used in humans for many years as a treatment for gout, by inhibiting the enzyme xanthine oxidase in the production of uric acid. Allopurinol does not inhibit human hypoxanthine-guanine phosphoribosyltransferase and, thus, does not cause Lesch-Nyhan type syndrome, known to be associated with the enzyme deficiency. Although *T.cruzi* infected mice treated with this drug showed significant increases in survival times when compared with controls (Avila and Avila, 1981), some *T.cruzi* strains are not responsive to this type of compound, which suggests that metabolic differences are present between the different strains (Marr *et al.*, 1978; Avila *et al.*, 1981, 1984). This raises doubts as to the efficacy of these drugs to treat Chagas' disease in man. More recently, allopurinol ribonucleoside and another inosine analogue, formycin B has been tested against Leishmania with some success (Nelson *et al.*, 1982). Research is continuing in order to



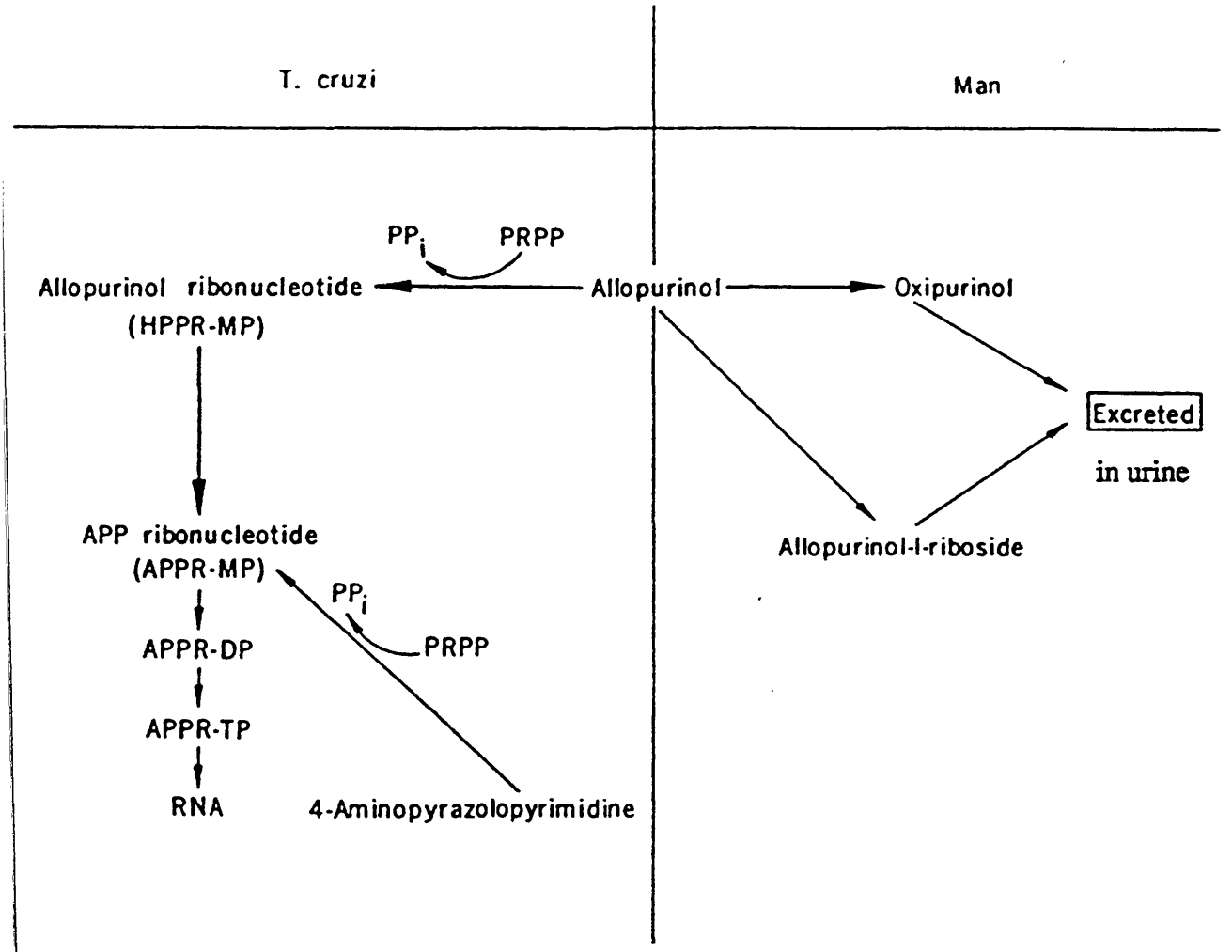


Figure 1.18. Structure of hypoxanthine and metabolic transformation of allopurinol in *Trypanosoma cruzi* (Morello, 1988).

Enzyme activities : (1) succino-AMP synthetase; (2) succino-AMP-lyase.

identify differences in metabolic pathways in the parasite which are distinct from the mammalian host in order to develop new antichagasic chemicals so badly needed.

Aims of the research project.

1. To determine the existence of a functional pentose phosphate pathway in the bloodstream form of *T.b.brucei* by studying the differential labelling of RNA in trypanosomes incubated with [^{14}C]glucose labelled at C-1 and C-6 positions, and to investigate whether the label is incorporated into the base and/or ribose moieties of RNA.

2. To purify, characterise and describe the physiological role of the enzyme PRPP synthetase in *T.b.brucei*, with particular emphasis on its localisation and possible interaction with other metabolic pathways, such as the salvage of nucleosides and bases and the pentose phosphate pathway in view of the presence of intracellular compartments in *T.b.brucei*.

CHAPTER 2

INCORPORATION OF RADIOLABEL INTO TRYPANOSOMAL RNA USING DIFFERENTIALLY LABELLED [^{14}C] GLUCOSE

2.1. Introduction.

A vast amount of knowledge has accumulated about the metabolism of glucose *via* the glycolytic pathway in African trypanosomes (see review by Opperdoes, 1987).

Very little is known about alternative routes of glucose metabolism in trypanosomes. The metabolism of glucose *via* the oxidative pentose phosphate pathway has been shown by Grant and Fulton in 1957 and Fairlamb and Opperdoes in 1986 not to be a major pathway in *T.brucei*. In 1962, Ryley reported the presence of glucose-6-phosphate dehydrogenase in *T.brucei* but was unable to detect 6-phosphogluconate dehydrogenase or Entner-Doudoroff pathway activity. The fate of 6-phosphogluconate was considered problematic until recently, when Cronin *et al.* in 1989, showed the presence of most of the enzymic components of the oxidative pentose phosphate pathway, including 6-phosphogluconate dehydrogenase.

In recent years there has been a lot of indirect evidence for the existence of an active pentose phosphate pathway in trypanosomes. Preliminary results from this laboratory (Hunt *et al.*, 1986), on incorporation of label into cells incubated with differentially labelled [^{14}C]glucose indicated that a total RNA fraction had a labelling pattern that was consistent with provision of pentose from an oxidative pentose phosphate pathway. Other work that suggests the operation of an oxidative pentose phosphate pathway includes: (a) Phosphoribosyltransferase activities for adenine, hypoxanthine and guanine was present in bloodstream forms of *T.brucei* (Davies *et al.*, 1983) which require PRPP as substrate which must have originated from a pentose phosphate precursor. (b) Procyclic forms of *T.gambiense* and bloodstream forms of both *T.gambiense* and *T.rhodesiense* were unable to synthesise purines *de novo* and suggested the operation of an interconverting purine salvage pathway based on the metabolic fate of isotopically labelled purine bases, nucleosides and various precursors (formate, glycine, serine and glucose) for purine nucleotide biosynthesis (Fish *et al.* 1982a, 1982b). The identification of the enzymes of *de*

novo pyrimidine synthesis in various species of trypanosomes suggests this hypothesis (Hammond and Gutteridge, 1984).

Substrates utilised and catabolic pathways present in mammalian forms of *T.cruzi* are similar to those of culture forms of *T.cruzi* and are quite distinct from those of the bloodstream form of *T.brucei* (Rogerson and Gutteridge, 1979). In 1959, Raw demonstrated the presence of the two primary enzymes of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and Mancilla and Naquira in 1964, showed that the pentose phosphate pathway contributes significantly towards glucose catabolism but the proportion varied with different strains used.

The following experiments confirmed the existence in bloodstream forms of *T.brucei* and in epimastigote forms of *T.cruzi* of a small, but significant proportion of supplied glucose being metabolised through an oxidative pentose phosphate pathway leading to provision of ribose-5-phosphate which is subsequently incorporated into RNA.

2.2.0. Materials.

Potassium dihydrogen orthophosphate, magnesium sulphate, potassium chloride, ethylenediaminetetraacetic acid (EDTA) disodium salt, sodium hydroxide, hydrochloric acid, perchloric acid, sulphuric acid, tris(hydroxy-methyl)aminomethane hydrochloride (Tris-HCl), Orcinol (3,5 dihydroxytoluene), Sarkosyl NL 30, formic acid, glacial acetic acid and D-glucose were Analar grade from BDH Chemicals Ltd, Poole, England.

Sucrose, glycerol, orthophosphoric acid, trichloroacetic acid and sodium chloride were Analar grade from Fisons plc., Loughborough, England.

Heparin (grade I, porcine intestinal mucosa) sodium salt, Antifoam A emulsion (30 % aqueous emulsion, a 100 % active silicone polymer with anionic emulsifiers), isoamyl alcohol, ferric chloride (anhydrous) were purchased from Sigma Chemical Company, St. Louis, Mo., U.S.A.

Ribonuclease (RNAse), glucose oxidase (GOD) and peroxidase (POD) were also purchased from Sigma. GOD was supplied as a lyophilised powder containing ~80% protein, with specific activity, 146 units mg^{-1}, where 1 unit is the amount of enzyme required to convert 1μmole of glucose to gluconic acid and peroxide min^{-1} at pH 5.1 and a temperature of 35°C. POD, R.Z.1.1, was supplied as a salt-free powder with specific activity of 95 units mg^{-1}, where one unit is the amount of enzyme required to convert 1mg of pyrogallol to purpurogallin and peroxide in 20sec at pH 6.0 and at a temperature of 20°C. The R.Z. is a measure of the haemin content of a peroxidase preparation.

Caesium chloride Analar grade (99.9 % minimum) was obtained from Rose Chemicals Ltd, London, England.

Ethanol, methanol and diethyl ether were supplied by James Burrough, England.

Anion exchanger diethylaminoethyl cellulose (pre-swollen DE-52) and cellulose filter papers 3MM (2.4 cm Diameter) were purchased from Whatman Ltd, Maidstone, Kent, England.

Anion exchange resin, Dowex 1-X8 (200-400 mesh) chloride form was purchased from Bio-Rad Laboratories, Richmond, Ca., U.S.A.

Optiphase 'Safe' Scintillation fluid was purchased from Pharmacia Biosystems Ltd, Milton Keynes, Bucks., England.

Sabre sterile hypodermic needles, 0.8 mm x 40 mm, were used for infection and bleeding into Gillette 20 ml plastic disposable syringes.

Pasteur pipettes and micro-haematocrit tubes were purchased from Bilbate, Daventry, England.

2.2.1. Radiochemicals employed.

Stocks of [U-^{14}C]glucose (270mCi mmole^{-1}), [1-^{14}C]glucose (3 and 55.6mCi mmole^{-1}) and [6-^{14}C]glucose (58.5mCi mmole^{-1}) were purchased from Amersham International plc., Buckinghamshire, England.

The nominal activities of radiolabelled glucose in the incubations were calculated from the specific activities of the stock solutions purchased and the volumes in which the radiochemicals were supplied. The radiochemicals were stored at -20°C.

2.2.2. Organisms used.

A. Mammalian host.

Adult male or female, Wistar and/or Sprague-Dawley rats of 200-800 g weight, were bred and supplied by the University of Bath animal house.

B. Parasitic forms used.

1. Bloodstream form of *Trypanosoma brucei brucei.*

Stocks of clones of long slender bloodstream forms of *T.b.brucei* strain EATRO 427-12/ICI-060 were kindly supplied by Dr. Paul Voorheis, Trinity College, Dublin, Ireland, and stored as frozen stabilates at -70°C.

2. Culture forms of *Trypanosoma cruzi.*

These were cultured and prepared by Tim Sharpington, London School of Hygiene and Tropical Medicine, London. The axenic culture contained RPMI medium suplemented with HEPES, trypticase, Hemin, glutamine, 10 % fetal calf serum and the antibiotics penicillin and streptomycin. The strain supplied was strain X10-1 and the culture medium seeded at a cell density of 1-2 x 10^7 cells ml^{-1} and incubated at 28°C. All procedures that involved live cells were carried out in plastic tubes under sterile conditions at the London School of Hygiene and Tropical Medicine to avoid infection.

2.3.0. Buffers and reagents.

2.3.1. Krebs-Ringer phosphate buffers.

2.3.2. Krebs-Ringer phosphate buffer (KRP).

This buffer was prepared according to Ryley,1955.

KH_2PO_4	22mM
NaCl	98mM
$MgSO_4$	1.0mM
KCl	2.0mM

The pH was adjusted to 8.0.

2.3.3. Krebs-Ringer phosphate buffer with sucrose and glucose (KRPSG).

Glucose	10mM (freshly added)
Sucrose	3 % (w v^{-1}) (freshly added)

2.3.4. Glucose incubation buffer.

KRP buffer containing 0.15 % (w v^{-1}) bovine serum albumin (Sigma fraction V), pH 8.0 and 20µl antifoam per 40ml buffer.

2.4.0. Nucleic acid extraction buffers.

2.4.1. Extraction (lysis) buffer.

Tris-HCl	100mM
NaCl	50mM
EDTA	5mM
Sarkosyl	1 % (w v^{-1}) (added after pH adjustment)

The pH was adjusted to 8.0.

2.4.2. TE buffer.

Tris-HCl	10mM
EDTA	150mM

The pH was adjusted to 8.0.

2.4.3. Sodium chloride-sodium citrate buffer (SSC buffer).

NaCl	1.5M
Sodium citrate	150mM

The pH was adjusted to 7.0.

2.4.4. Chloroform reagent.

$CHCl_3$:3 methyl-1-butanol (24:1,v v^{-1})

2.4.5. Phenol.

Phenol:water (80 %,w w^{-1}), dissolved on a hot-plate in a fume cupboard and redistilled.

2.4.6. Orcinol reagent.

Orcinol	1.0 % (w v^{-1})
$FeCl_3$	0.5 % (w v^{-1})

This was made up to the required volume with concentrated HCl.

2.5.0. Methods.

2.5.1. Preparation of glassware and reagent sterilisation.

Glassware which was used with crude and purified nucleic acid preparation and analysis was autoclaved at 150°C for 70min. Plasticware and solutions were sterilised at 121°C for 30min. This procedure denatured the ubiquitous RNAse activity found even on fingertips.

Gloves were used whenever handling of RNA and radiochemicals was involved.

2.5.2. Preparation of acid washed filter papers.

The Whatman 3MM filter papers were washed with 20 % ($w.v^{-1}$) trichloroacetic acid in a beaker for 30min and rinsed with diethyl ether, followed with ethanol and dried. The papers were marked on one side with an HB pencil.

2.5.3. Preparation of CO_2-free water.

CO_2-free water was prepared by vigorously boiling 500ml water with 10-15 anti-bumping granules on a hot plate. After 10min, the water was cooled having been stoppered with a tube filled with sodalime.

2.5.4. Preparation and storage of *T.b.brucei* stabilates.

Heparinised blood from a severely infected rat (10^9 cells ml^{-1} blood) was cooled on ice and glycerol was slowly added by gentle stirring with a glass rod to 10-15 % ($w\ v^{-1}$). About 20µl of blood was drawn by cappillary action into glass cappillary tubes and both ends sealed using the blue part of a Bunsen burner flame.

The glass cappillary tubes were very slowly cooled to -40°C by slowly introducing them into a flask of liquid-nitrogen fridge using a specially designed apparatus which fits into the neck of the liquid-nitrogen container. After 3-4

hours the stabilates were immersed in liquid-nitrogen where the cells retained their viability and infectivity for at least several years.

The contents of one or more tubes were suitably diluted in KRPSG isotonic buffer and injected intraperitoneally into an uninfected rat at a density of 1-3 10^7 cells per 200-400g rat.

2.5.5. Counting of trypanosomes.

The number of active mobile trypanosomes was determined using the chamber of an improved Neubauer haemocytometer (depth 0.1mm, volume of chamber 0.1mm^3) and the 40x objective of a Zeiss light microscope. The chamber is divided into 25 squares with a total area of 1.00 mm^2.

The cells in five, squares were counted. If N equals the number of the cells in the five squares, Nx5 equals the number of the cells in the chamber.

0.10mmx1.00mm^2 = 0.1mm^3

A volume of 0.1mm^3 contains Nx5 cells (if the cell suspension has been diluted this value must be multiplied by the dilution factor).

A volume of 1000mm^3 (1.0ml) contains:

(N x 5 x dilution factor x 1000) / 0.1 = N x 5 x dilution factor x 10^4 cells.

2.5.6. Preparation of anion-exchanger DE-52.

The pre-swollen microgranular ion exchanger DE-52 was equilibrated with KRP buffer in the ratio of 100g of pre-swollen adsorbent to 1.5l of KRP buffer.The adsorbent was allowed to settle for 20min and the supernatant, containing the fines, was sucked off. This was repeated five or six times and the sediment was resuspended in KRP buffer and the pH adjusted to 8.0 with HCl. This was stored in the cold room and used as required.

2.5.7. Preparation of the DE-52 column.

The column was prepared freshly on the day of trypanosome harvest.In a 20ml syringe, glass wool was placed over the hole and sufficient DEAE cellulose up to 8-10ml. The column was equilibrated with several volumes of KRPSG buffer.

2.5.8. Harvesting of trypanosomes.

The infection of the rat was allowed to proceed for 72 hours. The rats were anaesthetised using diethyl ether and bled into 20ml syringes containing 1ml of heparin solution (200 units ml^{-1}) in KRP buffer. Bleeding was carried out by insertion of the hypodermic needle into the bifurcation of the common iliac artery. Between 10 and 15ml blood was recovered from a 200g rat.

2.5.9. Purification of trypanosomes.

The trypanosomes were separated from the blood by differential centrifugation at 1000*g* at 4°C in an IEC CENTRA-3R refrigirated centrifuge and purified by the method of Lanham and Godfrey, (1970).

The blood was centrifuged for 5min at 1000*g*, at 4°C and the supernatant was discarded and the trypanosomes, which separated as a white layer above the red cells, were removed as completely as possible by means of a Pasteur pipette into KRPSG buffer. The trypanosome suspension was again centrifuged, the upper layer being collected and the small lower layer of contaminating red cells discarded. The process was repeated until most of the blood elements were removed.

Platelets, residual red blood cells and white blood cells were separated from the trypanosomes on the basis of the surface charge of many species of trypanosomes being less negative than that of the blood cells of rats and mice in the pH range 6-9 (Lanham, 1968).

The trypanosomes were purified by passage through a column (8-10ml) of DEAE-cellulose (Whatman DE-52) pre-equilibrated with KRPSG buffer at 4°C. The trypanosomes eluted from the column as a milky suspension with KRPSG buffer and washed once with KRPSG buffer. They were counted and kept on ice until required.

2.5.10. Glucose utilisation by bloodstream form of *T.b.brucei in vitro.*

Incubations were carried out at 37°C in a four-port 250ml flask in 36ml of stirred incubation buffer while gassing with pre-humidified air. The gas outflow was bubbled through 5ml of 1M NaOH in order to trap the evolved CO_2.

The incubation volume was brought up to 40ml by addition of $4x10^9$ cells in 4ml KRPSG buffer to give a final concentration of glucose of 1mM and a cell density of 10^8 cells ml^{-1}.

At certain time intervals , aliquots of 200µl were removed and 10µl diluted 100x in incubation buffer and the number of viable cells estimated. The remaining aliquot was quickly spun on a microfuge and the supernatant suitably diluted for glucose estimation.

2.5.11. Assay for Glucose estimation.

A modified version of the method of Bergmeyer (1974) was employed.

The buffer-enzyme mixture used for this assay contained 5.13mg/150ml GOD and 1.58mg/150ml POD in 98mM potassium phosphate buffer, pH 7.2.

To 150ml of the above buffer, 0.6ml of an o-dianisidine dihydrochloride solution containing 10mg ml^{-1} was added to form the assay reagent.

Aliquotes of incubation mixture were spun at 1000*g* for 5min and volumes of 25-50µl were made up to 0.5ml with water in test tubes. 5.0ml of assay reagent were added and the tubes were incubated at room temperature for 45min. The absorbance of the samples was measured against a blank (0.5ml of incubation buffer without any glucose plus 5.0ml of assay reagent) at 436nm with a 1cm light path in a Philips PYE-Unicam SP6-450 UV/VIS Spectrophoto-

meter. The glucose concentration was determined by comparison of the spectrophotometer readings with a standard curve prepared with glucose concentrations 0 to 20mg per 100ml. The maximum linear range of the assay is 15mg glucose per 100ml.

2.5.12. Incubation of *T.b.brucei* cells with [^{14}C]glucose.

The experimental set up was similar to the glucose utilisation system the only difference being that radiolabelled glucose was added to the flask. An aliquot of the incubation mixture was removed for the estimation of total glucose added.

After 25min a further 200µl. sample was taken and the contents of the flask transferred to 4 pre-cooled centrifuge tubes. The cells were pelleted at 1000*g* at 4°C for 5min. The supernatant was carefully poured off and the cells resuspended in a total of 8ml of KRP buffer and respun. The cell pellet was resuspended in 1ml of sterile KRP buffer and lysis of the cells was achieved with the addition of 4ml extraction buffer containing Sarkosyl detergent.

2.5.13. Incubation of *T.cruzi* cells with [^{14}C]glucose.

The incubation set-up was effectively that of the *T.brucei*, the difference being the absence of unlabelled glucose. The trypanosomes were introduced into the incubation medium in KRP buffer so the only glucose present in the system was the radioactive [^{14}C]glucose added directly to the flask. The final concentration of glucose was approximately 8.7µM. According to von Brand (1961) , these cells survive for long periods in the absence of glucose and can metabolise proteins and fatty acid reserves. Bovine serum albumin was also excluded, the incubation time extended to 150min and the incubation carried out at 28°C. All other procedures were exactly the same as for *T.brucei* cells.

2.5.14. Estimation of radioactivity of an aqueous sample.

A constant ratio of 1:10 (v v^{-1})of aqueous sample to scintillation fluid was used and a final volume of 5ml counted in 50ml super polypropylene vials for 10min per vial. The LKB Wallac 1217 Rackbeta liquid scintillation counter had a window of 0-156 KeV. As the counting efficiency of [^{14}C] counting was ≥95% all counts in cpm were not converted into dpm.

2.5.15. Estimation of the evolution of label as [^{14}C]CO_2.

An aliquot of 50µl from the 1M NaOH through which the gas outflow was passed was diluted 10x with water and twice the usual volume of scintillation fluid added in order to get a clear sample for counting. The vials were counted for 10min each and the total counts of [^{14}C] in the CO_2 estimated after multiplying the counts from the 50µl aliquot (corrected for background radioactivity using a water blank with the same ratio of sample to scintillation fluid) by the dilution factor of 100.

2.5.16. Nucleic acid extraction.

Nucleic acid extraction and mass protein removal were carried out simultaneously with phenol and chloroform reagent according to Pryke *et al.* (1979).

The cell preparation was stirred, 2ml phenol and 2ml chloroform reagent were added and the mixture gently stirred for 5min and then centrifuged at 800*g* for 5min in a glass tube. The top (aqueous) layer and the white interface were removed and re-extracted with 4ml chloroform reagent in a glass tube with quick-fit stopper for 5min. After spinning as before, the top layer was taken for two further extractions with chloroform reagent. The final top layer was used to prepare the caesium chloride density gradient.

2.5.17. RNA purification - CsCl density gradient centrifugation.

To the nucleic acid extraction volume, one-tenth volume of SSC buffer was added and solid CsCl 1.3:1 w v^{-1} was added.

The solution was placed in Beckman quick-seal ultracentrifuge tubes (5.2ml max volume per tube) and sealed by melting after carefully balancing against each other (less than 50mg difference).

The tubes were spun at 200,000*g* for 18 hours at 15°C in a vertical rotor (VTi 65) in a Beckman L5-65 ultracentrifuge. After deceleration without brake the top of the tube was sliced off with a razor blade and drained of liquid using a sterile Pasteur pipette. The RNA pellet which was clearly visible down the wall of the tube was allowed to dry and was resuspended in a small volume of sterile TE buffer. The suspension was made up to 0.4 M NaCl and 2 volumes of ethanol (absolute ethanol at -20°C) were added. The solution was stored overnight at -70°C. The RNA was recovered by centrifugation at 20,000*g* for 10 min at 2°C. The pellet was resuspended in a small volume of sterile TE buffer and aliquots taken for RNA determination, radioactivity estimation and acid hydrolyses.

2.5.18. Orcinol method for RNA determination.

The Orcinol method of Schneider (1957) was employed.

Volumes of RNA (75-150µl) were diluted to 0.5ml with TE buffer in duplicate. 0.5ml perchloric acid (72% v/v) and 1ml of Orcinol reagent were added and mixed. The test tubes were heated in a boiling water bath for 20min. After cooling, the absorbance at 660nm was measured against a reagent blank. A standard calibration curve was constructed using standard RNA (500µgml^{-1}) in TE buffer at 0-100µl ml^{-1} concentration in the assay. The assay was linear for up to at least 100 µg ml^{-1}.

2.5.19. RNAse assay to confirm the radioactivity of RNA.

Ribonuclease (RNAse), from *Aspergillus clavatus* supplied as lyophilised powder containing about 5% protein was used. The activity was approximately

250,000 units mg^{-1} protein, where one unit will produce acid soluble oligonucleotides equivalent to a DA_{260} of 1.0 in 30 min at pH 7.5 at 37°C in a 1.5ml reaction volume.

Excess RNAse (80 units ml^{-1}) free from DNAse and protease activities was used in the presence of 100μM PMSF. It was incubated with RNA for 2, 5 and 15 min at room temperature. The incubation buffer was 0.1 M acetate (pH 5.0). Control tubes with no RNAse were also set up. At the set time intervals, aliquots of 20μl out of a reaction volume of 100 μl were placed onto acid pre-washed filter papers and allowed to dry.

The filter papers (Whatman 3MM) were then washed 4 times with 5% TCA, once with ethanol and allowed to dry. Scintillation fluid (5ml) was added to each vial containing the filter paper and counted for 10 min.

The counts that remained on the filter papers represented the acid insoluble nucleic acid (RNA) that was not hydrolysed in the absence of RNAse in the control incubations.

2.5.20. RNA hydrolyses.

A. RNA hydrolysis with sulphuric acid.

Half the RNA stored as ethanol precipitate at -70°C was resuspended in 0.5 M H_2SO_4 and heated at 100°C for one hour. After cooling, the hydrolysate was subjected to anion-exchange column chromatography (See below).

According to Vischer and Chargaff (1948) , this hydrolysis is mild enough to prevent the breakdown of the pentose sugar component and so allow its chromatographic separation and quantitation.

From this hydrolysis free purine bases were obtained in addition to the ribose.

B. RNA hydrolysis with perchloric acid.

The rest of the RNA was mixed with 70% perchloric acid and heated at 100°C for 1 hour. The hydrolysate was diluted with two volumes of water and treated as for the sulphuric acid hydrolysate.

In this process, free pyrimidine bases were obtained while the ribose was charred.

2.5.21. Anion-exchange chromatography.

A formate Dowex 1-X8 column (mesh size 200-500) was prepared from a batch of chloride form by washing with 1 M NaOH followed by CO_2-free water, followed by 3 M formic acid and CO_2-free water. The column dimensions were 20cmx0.7cm diameter.

The hydrolysate (sulphuric or perchloric) was loaded onto the column and the column washed with water. In the case of the sulphuric acid hydrolysate the water eluant contained the ribose, adenine and guanine and in the case of the perchloric acid hydrolysate, the water eluant contained cytosine and uracil only.

The sample and water were passed through the column by a peristaltic pump, at a rate of 1 ml min^{-1}. The eluate tube was connected to a CECIL CE 272 linear ultraviolet spectrophotometer, set at 260nm, which was connected to a Rikadenki electronic recorder. The eluate was collected into a round-bottom flask which fitted onto a vaccum rotor evaporator which was used in evaporating the formic acid.

2.5.22. Evaporation of the formic acid - Preparation for TLC.

The flask was attached to a rotor vaccum evaporator which was attached to an oil vaccum pump. The sample was in the flask which was immersed in a constant temperature water bath at 48°C. Water up to twenty times the volume was added at intervals during the evaporation of the formic acid. When neutrality was reached, the volume was reduced to 100µl and was spotted in a line five cm long 2cm from the bottom of the silica gel 60 coated glass plate 10 x 20 cm.

Mixture of markers adenine, cytosine, guanine, uracil and ribose, 5μg each were spotted on either side of the eluate. The plate was developed at room temperature,about 22°C, in the following solvent, Acetonitrile : glacial acetic acid : ethanol : water (195 : 15 : 2 : 1 ; v : v : v : v). The plate was developed for 2 1/2 hours and dried at room temperature.

2.5.23. Quantitation of bases and ribose.

The positions of the bases were viewed under U.V. light and they appeared as dark areas against the overall green background fluorescence of the plate.

The positions were marked with a pencil and the plate cut using a diamond cutter to remove the marker columns either side of the eluate.

The bands were scraped carefully and eluted with water overnight in 1ml per 1x5cm^2 area. The tubes were mixed occasionaly to aid elution. The silica was spun down in a bench centrifuge and the supernatant used for spectral characterisation and calculation of the concentration of the bases from their respective wavelength maxima in 0.1M HCl after the subtraction of a TLC blank (Burton, 1969). An aliquot was taken for estimation of the radioactivity and the specific molar radioactivity of each base calculated.

Ribose was located on the plate by charring with sulphuric acid in methanol (5%, v v^{-1}) using a Bunsen burner flame. This was only carried out on the marker columns to avoid quenching and to ensure the quantitative estimation of the ribose by an adapted Orcinol method (Mejbaum, 1939). The eluate ribose was scraped and a suitable blank included.

The R_f value of each component was calculated as follows:

$$R_f = \frac{\text{distance travelled by component}}{\text{distance travelled by solvent}} \times 100$$

The R_f values for the four bases and the ribose are presented in Table 2.1.

The specific activity of radioactivity was calculated from a small fraction of supernatant not used for the quantitation of the ribose.

2.5.24. Quantitative assay for ribose.

The method used is a variation of the assay for RNA, the difference being in that instead of perchloric acid, water was added. The calibration curve was constructed using standard ribose (0.1 mg ml^{-1}) in water at 0-50 $\mu g\ ml^{-1}$ concentration in the assay. The assay was linear for up to 50 $\mu g\ ml^{-1}$.

2.5.25. Estimation of the ribose content of RNA.

This estimation assumes that the four nucleic acid bases had equal distribution in the RNA. The relative molecular mass of RNA was then calculated as the arithmetic mean of the molecular masses of the four anhydrous mononucleotides.

Adenosine 5'phosphate	347.2
Cytidine 5'phosphate	323.2
Guanosine 5'phosphate	363.2
Uridine 5'phosphate	324.2

RNA component	R_f value
Adenine	0.430
Cytosine	0.378
Guanine	0.016
Uracil	0.730
Ribose	0.522

Table 2.1. R_f values of ribose and the four nucleic acid bases of RNA obtained with pre-coated silica gel TLC plates using solvent acetonitrile : glacial acetic acid : ethanol : water (195 : 15 : 2 : 1, v/v)

The average relative molecular weight of a mononucleotide constituent of RNA is therefore equal to 339.45.

Ribose 150.0

$$\% \text{ Ribose in RNA} = \frac{150.00}{339.45} \times 100 = 44.19\%$$

One 100 equivalents of RNA contain approximately 44.2 equivalents of ribose.

2.6.0. Results.

2.6.1. Glucose utilisation by bloodstream form of *T.b.brucei.*

The *in vitro* survival of the bloodstream form of *T.b.brucei* is dependent on a readily available carbohydrate supply (Fulton and Stevens 1945). Under the specified conditions of *in vitro* incubation of $4x10^9$ cells in 1 mM glucose, it was found (Figure 2.1) that all the glucose was metabolised within 40 minutes of incubation. Cell death started at a glucose concentration of ~0.05 mM and after 40 minutes ~27% of the cells had died.

From this result it was decided that all subsequent incubations would end at 25 minutes after which ~94% of the glucose supplied had been metabolised and all the cells were still viable.

2.6.2. Incubation of bloodstream form of *T.b.brucei* with [^{14}C]glucose.

There was very little difference in the radioactivity of the incubation medium at the start and in the end of the incubation period with *T.b.brucei* This was due to the catabolism of radioactive glucose resulting in the extracellular excretion of radioactive products, mainly pyruvate and a small amount of glycerol (Grant and Fulton, 1957).

2.6.3. Incubation of cultured epimastigote form of *T.cruzi* with [^{14}C]glucose.

Carbohydrate metabolism in *T.cruzi* epimastigotes and blood trypomastigotes resembles that of the insect stage of *T.b.brucei* rather than that of the bloodstream form, in that carbon dioxide, succinate and acetate are produced as end-products (Bowman 1974).

Culture epimastigote forms of *T.cruzi* can survive incubation in Krebs-Ringer solution without added glucose for 24 hours. During starvation, triglycerides are broken down to free fatty acids and they appear to be the free energy reserves of these organisms (Rogerson and Gutteridge, 1980).

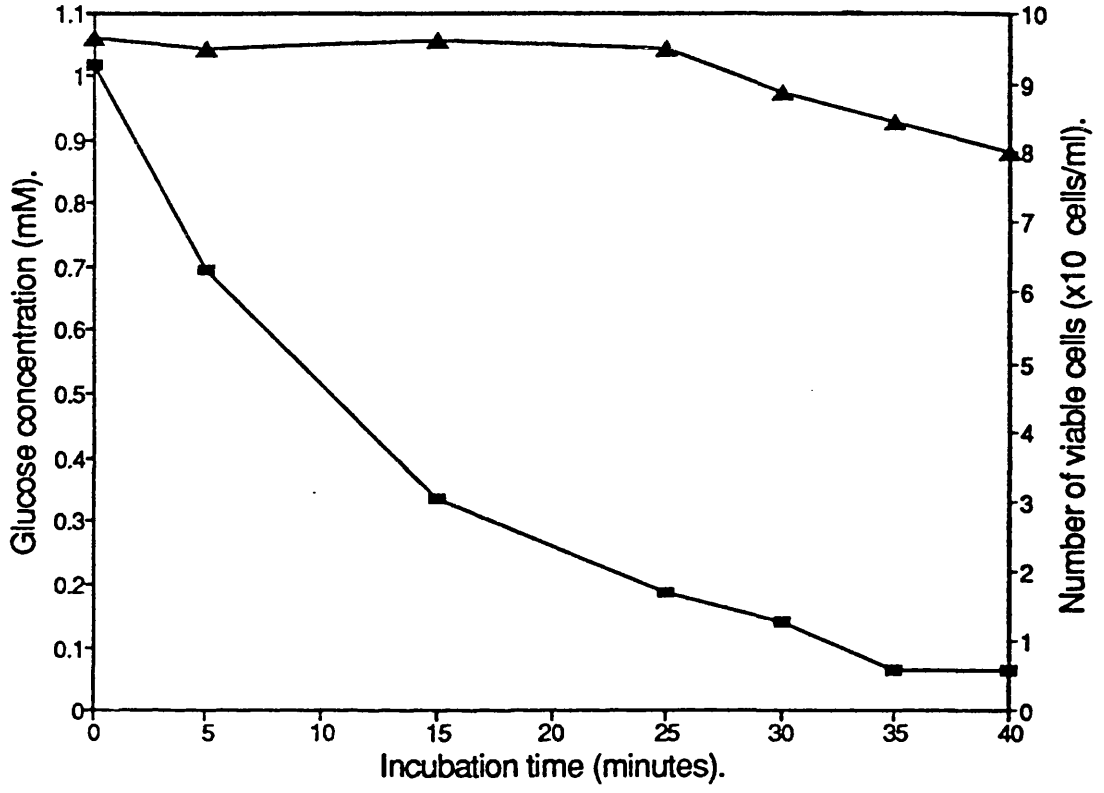


Figure 2.1. Glucose utilisation and survival of bloodstream *T.b.brucei* cells incubated at 37°C.

■——■ Glucose concentration in the medium (mM)

▲——▲ number of viable cells (x 10^{-7} cells ml^{-1}).

There was a bigger difference in the radioactivity of the incubation medium before and after the incubation than in the *T.brucei* incubations. This is because a larger fraction of the label was lost as $^{14}CO_2$.

2.6.4. Evolution of label in CO_2.

The incorporation of labelled glucose into CO_2 is presented in Tables 2.2 and 2.3 for incubations with *T.b.brucei* and *T.cruzi* cells respectively

With *T.b.brucei*, in all the incubations, there was a small percentage incorporation of total label metabolised into CO_2. There is a >10-fold difference in the total counts recovered in CO_2 from [1-^{14}C] glucose than from [6-^{14}C]glucose. An even greater percentage of total counts was recovered in CO_2 from the [U-^{14}C]glucose incubation than from both the [1-^{14}C]glucose and [6-^{14}C]glucose incubations added together.

With *T.cruzi*, the percentage of total counts recovered in CO_2 was a lot higher than that with *T.b.brucei* in all the incubations. The highest incorporation was observed in the [1-^{14}C]glucose incubation, where ~14% of the total label supplied was recovered in CO_2. This value was ~2.5-fold higher than the value from [6-^{14}C]glucose. In contrast with *T.b.brucei*, the percentage of total counts recovered in CO_2 from [U-^{14}C]glucose metabolism was lower than that from the other two put together.

2.6.5. The incorporation of label into RNA and its ribose component.

Table 2.2 presents the specific activities of RNA and ribose in the incubations of *T.b.brucei* with differentially labelled [^{14}C]glucose. The specific activities of ribose were almost equal to those of the RNA from which it was derived. The difference in the values could be due to incorporation of label into the nucleic acid components of the RNA, or also due to a consistent error in the estimation of the quantities of RNA and ribose. The specific activity of RNA from [6-^{14}C]glucose was higher than that from the other incubations. The lowest specific activity of RNA was obtained from [1-^{14}C]glucose incubation. In order

Labelled glucose supplied	Total counts added ($\times 10^{-7}$) cpm	Specific activity of glucose ($\times 10^{-6}$) cpm/µmole	Total counts recovered in CO_2 ($\times 10^{-5}$) cpm	Percentage of total counts recovered in CO_2	Specific activity of RNA cpm/µmole	Specific activity of ribose cpm/µmole
[U-^{14}C]	5.976	1.494	2.151	0.360	6555	5790
	5.160	1.290	1.863	0.340	4642	4383
	12.143	1.518	4.095	0.340	3628	2986
[6-^{14}C]	5.089	1.272	0.051	0.010	10038	9485
	5.723	1.430	0.034	0.006	11030	9903
[1-^{14}C]	9.960	2.490	11.659	0.110	1690	1596
	9.000	2.250	9.760	0.110	2023	1857

Table 2.2. Incorporation of labelled glucose into CO_2 and specific activities of RNA and ribose from *T.b.brucei* following incubation of 4×10^9 cells as described in the text.

Labelled glucose supplied	Total counts added ($\times 10^{-7}$) cpm	Specific activity of glucose ($\times 10^{-8}$) cpm/µmole	Final glucose concentration in the medium (µM)	Total counts recovered in CO_2 ($\times 10^{-5}$) cpm/µmole	Percentage of total counts recovered in CO_2	Total counts recovered in RNA ($\times 10^{-6}$) cpm/µmole	Specific activity of RNA ($\times 10^{-6}$) cpm/µmole	Percentage molar incorporation into RNA
[U-^{14}C]	21.600	6.194	8.72	20.40	9.45	1.0010	1.46	0.236
[6-^{14}C]	4.367	1.252	8.72	24.05	5.50	0.1690	0.23	0.186
[1-^{14}C]	4.280	1.234	8.67	59.92	14.00	0.0957	0.12	0.100

Table 2.3. Incorporation of labelled glucose into CO_2 and RNA from 150 minute incubation at 28° C of 4 x 10^9 cultured epimastigote *T.cruzi* cells.

to correct for the different specific radioactivities of the labelled glucose in different experiments, the results were expressed as percentage molar incorporations (see Table 2.4), which were calculated as 100 x [specific activity of RNA (cpm $\mu mole^{-1}$)/ specific activity of glucose (cpm $\mu mole^{-1}$)]. The molar incorporations into ribose and RNA are approximately the same for each labelled glucose, and the amount of incorporation from [6-^{14}C]glucose incubation is roughly half an order of magnitude higher than that from [1-^{14}C]glucose incubation. The percentage molar incorporation into ribose and RNA from [U-^{14}C]glucose incubation is slightly less than half that from [6-^{14}C]glucose incubation.

The RNA results for three representative experiments with *T.cruzi* are presented in Table 2.3. Here the percentage molar incorporation of label into RNA from [U-^{14}C]glucose incubation is ~25% higher than from [6-^{14}C]glucose incubation. The percentage molar incorporation into RNA from [6-^{14}C]glucose incubation is ~20% higher than from [1-^{14}C]glucose incubation. These incubations were carried out using different amounts of glucose and in all the experiments almost all the label in the RNA was due to its ribose component.

2.6.6. The incorporation of label into the nucleic acid component of the RNA.

The data in Table 2.4 show that there was no incorporation of label into the purine bases of RNA in all the incubations of *T.b.brucei*. Although there was some incorporation of label into the pyrimidine bases, the values were very low and the contribution to the total label in the RNA is minimal. The quantities recovered were sufficient for accurate quantitation [Figures 2.2(1-4)] but the corresponding counts were too few for accurate determinations of specific radioactivity.

The data for the nucleic acid bases of *T.cruzi* RNA were also very low (percentage molar incorporation for pyrimidines <0.003) and they were considered too inaccurate to be presented. The purines were also slightly

		Percentage molar incorporation into:					
Labelled glucose supplied	Specific activity of glucose ($\times 10^{-6}$) cpm/μmole	RNA	Ribose	Adenine	Cytosine	Guanine	Uracil
[U-^{14}C]	1.494 1.290 1.518	0.330 0.360 0.240	0.330 0.340 0.200	0 0 nd	0.006 0.011 0.008	0 0 nd	0.004 0.007 0.005
[6-^{14}C]	1.272 1.430	0.790 0.770	0.740 0.690	0 nd	0.003 0.025	nd nd	0.023 0.028
[1-^{14}C]	2.490 2.250	0.068 0.089	0.064 0.082	nd 0	0.001 0.002	nd nd	0.003 0.010

Table 2.4. Incorporation of labelled glucose into RNA and its components from *T.b.brucei* (See table 2.2).

nd = not determined.

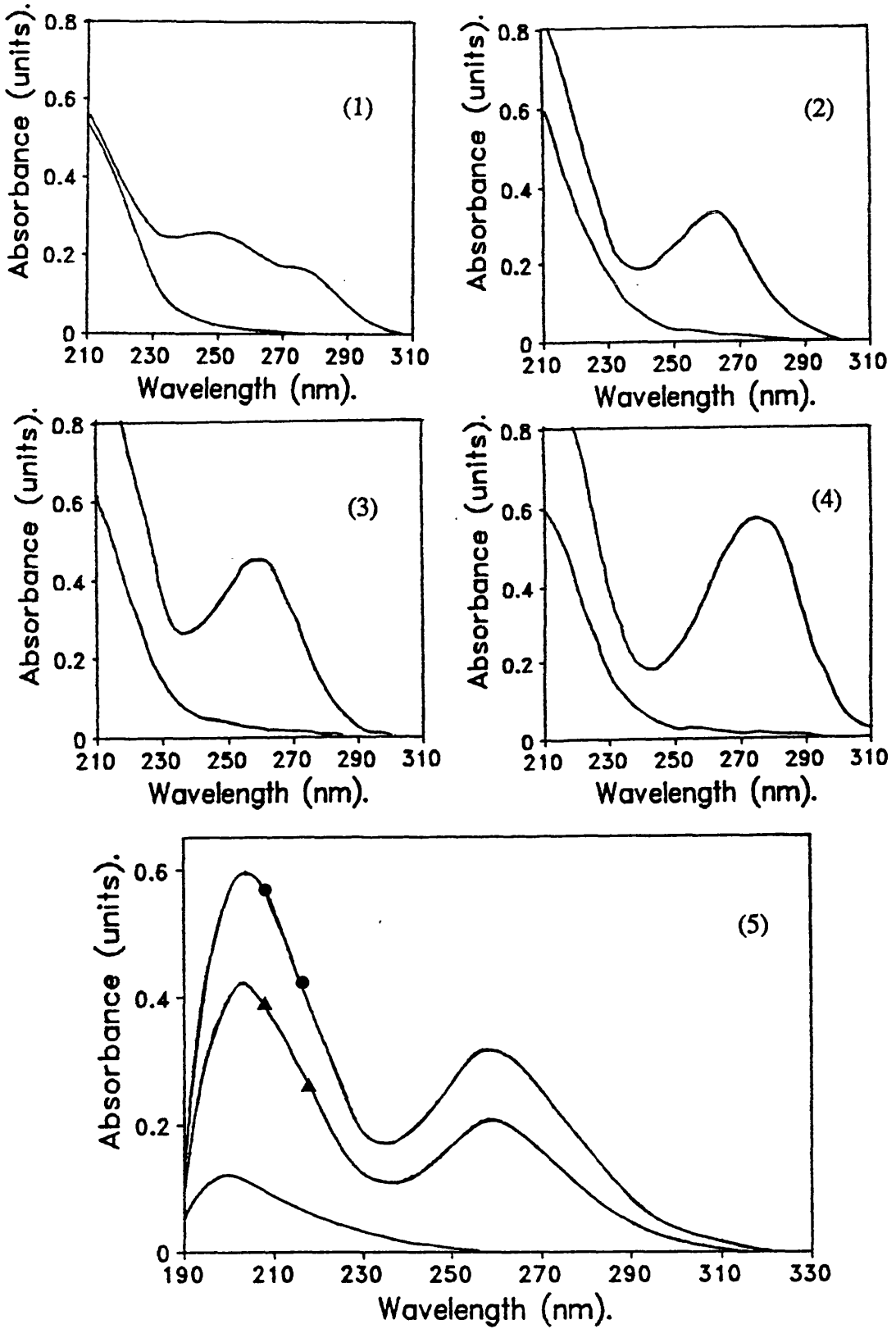


Figure 2.2. Absorbance spectra of bases: (1) guanine; (2) adenine; (3) uracil; (4) cytosine; (5) RNA; yeast RNA (●—●), trypanosome RNA (▲—▲) and buffer blank (——).

labelled (<0.1%) and the lack of contamination could not be excluded and therefore the results are not presented.

2.6.7. RNAse assay to confirm the radioactivity of RNA.

All insoluble radioactivity was removed from a TCA precipitate incubated with RNAse (10μg ml^{-1}) thus confirming that all the counts incorporated were in RNA.

The RNA yield from $4x10^7$ cells ranged between 600-1000 μg in total for *T.brucei*, the same number of *T.cruzi* cells gave on average half the RNA yield. The absorbance spectra of yeast RNA and the extracted purified trypanosomal RNA are presented in Figure 2.2(5).

2.7. Discussion.

African trypanosomes like *T.brucei*, are well adapted to life in the mammalian bloodstream. For their energy needs they rely entirely on glycolysis, which proceeds at a very high rate and leads to pyruvate excretion as the only end-product of aerobic glycolysis (see review by Brohn and Clarkson, 1978). Pyruvate cannot be further metabolised due to the absence of a functional tricarboxylic acid cycle (Fulton and Spooner 1959, Flynn and Bowman 1973) and the enzyme lactate dehydrogenase (Dixon 1966). In the absence of a readily metabolisable energy source *T.brucei* rapidly lose motility and lyse due to the lack of energy stores, as noted by Ryley in 1962.

In the present work, the rate of glucose consumption was monitored with time so that the cells were incubated for the longest period required to consume as much of the glucose supplied without loss of viability to the trypanosomes. The cell viability was followed by the mobility of the cells under microscopic observation. Although total cell viability and motility was maintained over the incubation periods, no cell proliferation occurred, as was shown by the constant number of cells present. In these experiments the incubation media did not contain any labelled nucleic acid bases or precursors which could have served as raw materials in the turnover of the trypanosomal RNA. Any incorporation of radioactivity in that pool would have arisen from *de novo* biosynthesis of nucleic acid bases or ribose.

In all the incubations, there was no incorporation of label from [U-^{14}C]glucose into the purine bases of the RNA, confirming the absence of *de novo* purine biosynthesis in *T.brucei* (Berens *et al.*, 1981). The functioning of various purine salvage pathways is therefore essential to the parasite's survival and growth *in vivo*. It was shown by James and Born in 1980 that the bloodstream forms of *T.brucei* can take up various nucleosides and bases from the host's fluids and incorporate them into its own nucleic acid biosynthesis.

The epimastigotes of *T.cruzi* are also unable to biosynthesise purines *de novo*, as suggested in previous experiments by Gutteridge and Gaborak (1979).

The required purine bases and nucleosides are salvaged from the host through the action of a range of phosphoribosyltransferases, aminohydrolases, kinases, hydrolases and phosphorylases (Gutteridge and Davies, 1981).

Incorporation of label from glucose into RNA must therefore have arisen from pyrimidine biosynthesis or via the pentose moeity. All six enzymes of *de novo* pyrimidine biosynthesis have been detected in homogenates of the blood trypomastigote form of *T.brucei* and the culture epimastigote form of *T.cruzi* (Gutteridge and Gaborak, 1979). Trypanosomes can also utilise salvage pathways for pyrimidine nucleotide synthesis (Hammond and Gutteridge, 1984). In all incubations the incorporation of label into the pyrimidines was very low. Because of this, and the low specific activities, no conclusions could be reached about the metabolic sourse of cytosine and uracil.

Previous work in this laboratory by Hunt *et al.* in 1986, had indicated the operation of a C-1 oxidative pathway leading from glucose to RNA in *T.brucei.* Recent detailed study by Cronin *et al.* in 1989, has shown the presence of most of the enzymic components of this pathway, including 6-phosphogluconate dehydrogenase long thought to be absent due to Ryley's inability to detect it in 1962.

Gutteridge and Gaborak (1979) have shown that none of the three forms of *T.cruzi* incorporated radiolabelled formate, bicarbonate, or glycine into the purines or their nucleic acids. Extracellularly supplied adenine and guanine were nevertheless incorporated into the nucleic acids as the respective nucleotides. Berens *et al.* (1981) used the sensitive technique of HPLC analysis of the soluble nucleotide pool of epimastigotes of *T.cruzi* to show that the observed incorporation of radioactivity in the nucleotide pool was not in the purine ring but in the ribose moiety. The demonstration that radioactivity from [U-^{14}C]glucose was incorporated, as the ribose moiety, into purine and pyrimidine ribonucleotides showed that these compounds were being made under the experimental conditions and that *T.cruzi* possesses adequate pentose phosphate shunt activity to supply the ribose, as PRPP, necessary for biosynthesis of nucleotides from

salvaged bases. Due to the overall low counts obtained with trypomastigotes of *T.brucei*, the experiments were extended to *T.cruzi* epimastigotes where at least the oxidative part of the pentose phosphate pathway has been identified by the presence of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities (De Boiso and Stoppani, 1973).

The higher percentage of glucose released as $^{14}CO_2$ in all the *T.cruzi* experiments compared to *T.brucei* is consistent with the well-known fact that in *T.cruzi* epimastigotes carbohydrate oxidation is *via* glycolysis, followed by a complete tricarboxylic acid cycle (Rogerson and Gutteridge, 1979). The yields of $^{14}CO_2$ from [1-^{14}C]glucose and [6-^{14}C]glucose of 14% and 5.5% respectively indicated that the hexose monophosphate shunt was contributing significantly to the metabolism of glucose. The yield of $^{14}CO_2$ from [U-^{14}C]glucose of 9.45% was lower than that quoted by Bowman *et al.* (1963), of 32%, which could reflect the switching-on of different pathways due to a lower concentration of glucose supplied in the present experiments. The understanding of the different pathways of glucose metabolism which operated in these experiments would need more scrupulous investigation involving the isolation and degradation of ribose and other end-metabolites such as succinate and acetate produced through CO_2-fixation. In order to attempt any calculation of the contribution of the pentose carbon cycle in the metabolism of glucose the labelling pattern of the trioses and other end-metabolites should be known.

The ratio of the percentage molar incorporations of radiolabel from glucose into the RNA is the inverse from that in the $^{14}CO_2$, consistent with the activity of the pentose phosphate pathway. The label from [U-^{14}C]glucose is ~25% higher than from [6-^{14}C]glucose. One might expect a lower result (5/6) if the only pathway was the pentose phosphate pathway, but the results suggest different pathways operating such as recycling of carbon and possible loss of C-6 before pentose synthesis.

In the *T.brucei* experiments, the ratio of radiolabel recovered in $^{14}CO_2$ from [1-^{14}C] and [6-^{14}C]glucose is indicative of a very active pentose

phosphate pathway. The higher $^{14}CO_2$ recovery from [U-^{14}C]glucose indicates loss of label through decarboxylation of other carbons such as [3,4-^{14}C] as has already been reported by Grant and Fulton in 1957.

There are formidable problems encountered in the attempt to calculate the contribution of the pentose phosphate pathway to the overall glucose metabolism using radiotracers. Most early attempts are discussed in reviews by Wood (1955), later refined by Katz and Wood (1960, 1963), Katz and Rognstad (1967) and many others. These methods are based on estimates from determinations of the fate of the labels of specifically labelled glucoses in their metabolism to various metabolic products. The yields of ^{14}C in CO_2 from labelled glucose are easily measured but are difficult to evaluate in terms of the metabolic pathways. All of the factors which affect the yields of ^{14}C in the triose phosphate come into play and superimposed on them are other factors. This is due to the fact that the pentose cycle yields CO_2 directly from C-1 of glucose *via* 6-phosphogluconate whereas the Embden-Meyerhof pathway yields none (Katz and Wood 1960). The principal purpose of the 1963 Katz and Wood paper was to consider procedures that utilise the yields of $^{14}CO_2$ from [1-^{14}C] and [6-^{14}C]glucose as a measure of the contribution of the different pathways of metabolism. A number of assumptions are made which are not necessarily valid when applied directly to any given system.

The following expression is based on the assumptions that the glucose supplied in the *T.brucei* experiments is solely metabolised via the Embden-Meyerhof pathway and the oxidative part of the pentose phosphate pathway. A second assumption is that the same amount of glucose supplied was metabolised in identical experiments irrespective of the label in the supplied glucose. Given that 94% of the glucose supplied in each case was metabolised in 25 minutes, the $^{14}CO_2$ yields from [1-^{14}C] and [6-^{14}C]glucose were expressed as specific yields, designated as $G1CO_2$ and $G6CO_2$, respectively.

In the [6-^{14}C]glucose experiment, the amount of glucose utilised was 5.089×10^7 cpm x 0.94, the yield of $^{14}CO_2$ recovered was 0.051×10^5 cpm.

$$G6CO_2 = \frac{0.051 \times 10^5}{4.784 \times 10^7} = 1.066 \times 10^{-4}$$

Similarly,

$$G1CO_2 = \frac{11.659 \times 10^5}{9.362 \times 10^7} = 0.01245$$

As was shown by Katz and Wood in 1963, when glucose is metabolised entirely by the pentose phosphate pathway and the glycolytic pathway,

$$\frac{G1CO_2 - G6CO_2}{1 - G6CO_2} = \frac{3PC}{1 + 2PC}$$

where, PC is the fraction of the total glucose that is metabolised *via* the pentose phosphate pathway.

By substituting the values for the specific yields calculated and solving the expression for PC the value of 0.415% is reached. This is a small yet significant amount of glucose metabolised *via* the pentose phosphate pathway under the conditions of these *in vitro* experiments. The concentration of glucose in the system is likely to affect the relative contribution of the various pathways operating and since these experiments do not involve cell proliferation it is likely that under *in vivo* conditions the need for ribose for RNA synthesis will switch-on a higher pentose phosphate pathway involvement in the overall glucose metabolism of the bloodstream form of *T.brucei*.

In *T.brucei*, a number of glycolytic enzymes present in the cytosol of other organisms are uniquely segregated in a microbody-like organelle, the glycosome, which they are believed to reach post-translationally after being synthesised by free ribosomes in the cytosol (Opperdoes *et al.*, 1986). Continuous and pulse-chase labelling experiments with procyclic trypomastigotes revealed that the enzymes have a half-life in the cytosol of about 3 minutes or less, and then turn over rapidly in the glycosomes, with half-lives as short as 30 minutes (Hart *et al.*, 1987). Some of the glycolytic enzyme activities associated with glycosomes such as TIM, GAPDH, PGK and ALDO all have cytosolic counterparts (Opperdoes *et al.*, 1986; Misset *et al.*, 1987). Under aerobic conditions, the function of the glycosome is intimately associated with that of the mitochondrial glycerol 3-phosphate oxidase, so that the overall reaction catalysed within the glycosome is:

$$\text{glucose} + 2\text{Pi} + O_2 \dashrightarrow 2(\text{3PGA}) + 2H_2O$$

The conversion of glucose to 3PGA proceeds with no net gain in ATP or NAD. The major function of the glycosome appears to be the rapid and efficient conversion of glucose or glycerol to 3PGA for ATP synthesis by pyruvate kinase in the cytosol. The reduced coenzyme, NADH, is indirectly re-oxidised by O_2 *via* the G3P-DHAP shuttle which involves speculation that specific translocators for DHAP, G3P, PGA, and Pi facillitate the diffusion of these phosphorylated species through an otherwise impermeable glycosomal membrane (Opperdoes and Borst, 1977). The current available evidence discussed by Fairlamb (1989) , supports the hypothesis that the glycosomal membrane acts as a barrier to diffusion, rather than a selectively permeable membrane.

The presence of cytoplasmic phosphoglucose isomerase in the bloodstream form of *T.brucei* is puzzling in view of the absence of transketolase in bloodstream forms (Cronin *et al.* , 1989), which is required in the metabolism of GAP to 3PGA in the incorporation of C-1 of glucose into ribose. The results

of Table 2.2 suggest that carbons 4, 5, and all of 6 find their way into the ribose since loss of carbons 1, 2, and 3 would explain the consistent lower molar incorporation from [U-^{14}C] than from [6-^{14}C] into the RNA. Mackenzie *et al.* (1982) incubated bloodstream forms of *T.b.gambiense* with [1-^{13}C]glucose and [1-^{13}C]glucose and assessed intracellular metabolic processes by NMR spectroscopy. They found that there was under anaerobic conditions predominant incorporation of C-1 of glucose into glycerol while there was complete randomisation of C-6 of glucose into glycerol and pyruvate. In later studies however, these workers (Mackenzie *et al.*, 1983) showed that there was a 2 to 1 ratio in the incorporation of C-6 of [6-^{13}C]glucose into C-3 of pyruvate, alanine, and 2,2-dihydroxypropionate (collectively) over C-1,3 of glycerol. The observations of a number of scientists such as Mackenzie *et al.* (1982), cannot be reconcilled by the Embden-Meyerhof glycolytic pathway and an alternative pathway of glucose catabolism must exist. Cronin *et al.* (1989), suggested that there could be a 2-carbon transfer independent of transketolase and 3'-epimerase activities which is yet unknown.

The complexity and indispensability of carbohydrate, mainly glucose, metabolism justifies research effort in evaluating the different pathways involved in the search of possible targets of chemotherapy against Trypanosomiases. The important result of this section of the present work has been the confirmation of the existence in the bloodstream forms of *T.brucei*, as well as in the epimastigote forms of *T.cruzi*, of a small, but significant, proportion of glucose metabolism through an oxidative pentose phosphate pathway leading to the provision of ribose moieties for subsequent incorporation into RNA.

CHAPTER 3

PRPP SYNTHETASE FROM *T.B.BRUCEI.*

3.1. Introduction.

The experiments of the previous chapter have shown that ribose is synthesised and incorporated into the RNA pool of *T.b.brucei* cells during the *in vitro* incubation of the cells with differentially labelled [^{14}C]glucose. The supply of ribose-5-phosphate into purine, pyrimidine and pyridine nucleotides takes place uniquelly through the catalytic action of the enzyme PRPP synthetase.

It is well reported in the literature that trypanosomes salvage purine and pyrimidine bases from their environment through the action of various phosphoribosyltransferases which require PRPP as one of their substrates (Fish *et al.*, 1982a,b). It was therefore interesting to detect and characterise the enzyme uniquely responsible for formation of PRPP in trypanosomes. In this chapter, the various methods used in the detection, partial purification and characterisation of PRPP synthetase from bloodstream forms of *T.b.brucei* are presented. The results obtained are compared with results from PRPP synthetases from different sources.

The assignment of the last two enzymes of *de novo* pyrimidine biosynthesis as well as of the purine salvage enzyme HGPRTase to the glycosome (Opperdoes, 1990), made the investigation of the intracellular localisation of trypanosomal PRPP synthetase very interesting.

3.2.0. Materials.

Additional materials to those used in chapter 2 were supplied as presented below.

Phenylmethylsulfonyl fluoride (PMSF), glycine, Brilliant Blue G (Coomassie Brilliant Blue G), High molecular weight marker proteins, triethanolamine hydrochloride, 2-mercaptoethanol, cupric chloride (dihydrate), nickel chloride (hexahydrate), ammonium chloride, streptomycin sulphate, N,N,N',N'-Tetramethylethylenediamine (TEMED), DL-Dithiothreitol (Cleland's reagent; DTT), 5-phosphorylribose-α-1-pyrophosphate (phosphoribosyl pyro-

phosphate, PRPP; estimated purity 80-90% based on the orotate removal assay), orotidine-5-monophosphate (OMP), orotic acid, inosine-5-monophosphate (IMP), hypoxanthine, adenosine, N_5',N'-Methylene-bis-Acrylamide, triethanolamine, Triton X-100, Blue Dextran (average molecular weight of 2,000,000), carbonic anhydrase, alcohol dehydrogenase, β-amylase, apoferritin, thyroglobulin, D-ribose-1-phosphate (cyclohexylammonium salt), D-ribose-5-phosphate (disodium salt), D-ribulose-5-phosphate (sodium salt), uridine-5-triphosphate (sodium salt), adenosine-5-diphoshate (sodium salt), 2-deoxyadenosine-5-triphosphate (disodium salt), cytidine-5-triphosphate (sodium salt), guanosine-5-triphosphate (sodium salt), inosine-5-triphosphate (sodium salt) were purchased from Sigma Chemical Company.

ATP (disodium salt) was purchased from Sigma and was purified from ADP and Pi contaminants using ion-exchange chromatography on a Dowex 1-X-8 (formate form) column, according to the method of Bartlett, 1959. The concentration of the eluted ATP solution was estimated from the extinction at 259nm; the molecular extinction coefficient was taken to be 15.4×10^3 at pH 7.0 (Glynn and Chappell, 1964).

Inorganic Pyrophosphatase (Pyrophosphate phosphohydrolase, PPiase; EC 3.6.1.1) from Bakers Yeast purchased as lyophilised powder from Sigma, and kindly donated by Dr. W. Whish, Biochemistry Department, University of Bath. It was supplied as 100 units, 0.2 mg protein/vial and 520 units/mg protein. One unit will liberate 1μmole of inorganic orthophosphate per minute at pH 7.2 at 25°C.

Orotidine-5'-phosphate pyrophosphorylase and Orotidine-5'-phosphate decarboxylase, (OPRTase-ODCase; mixed enzymes) were also from Sigma. These were supplied as a crude powder from Yeast, which contained ~50% buffer salts. Activity ~0.2-0.3 Units per mg solid. [One Unit will catalyse the phosphorylation of 1μmole of orotic acid to 5'-OMP, which is then decarboxylated to 5'-UMP in one hr at pH 8.0 at 25°C, in a PRPP system] .

HGPRTase purchased fromSigma was supplied as 1,000 mUnits. The white powder was dissolved in 1ml water and aliquotes of 80µl stored at -20°C.

Acrylamide, magnesium chloride (hexahydrate), sodium azide, ammonium persulphate, Bromophenol Blue, Eriochrome black T (Solochrome black), ammonia solution (sp. gravity 0.88g/cm^3), Tris(hydroxymethyl)-methylamine, butan-1-ol, zinc chloride, cobalt chloride (hexahydrate), sodium dodecyl sulphate (SDS), calcium chloride (hexahydrate), manganous chloride (tetrahydrate), polyethylene glycol (20,000) from BDH Chemicals Ltd.

Dialysis tubing (membrane), visking size 1-8/32" from Medicell International, London, U. K.. Ion-exchange paper DE-81 filter discs (2.3 cm diameter) from Whatman Ltd. Thin Layer Chromatography Polygram cel 300 PEI (0.1mm) pre-coated plastic sheets from Camlab Ltd. , Duren, Germany. Cibacron Blue F3G-A from Ciba-Geigy, Basel, Switzerland.

Bio-Rad protein assay (dye reagent concentrate) and Vertical slab gel apparatus (Proteam TM 11) from Bio-Rad Laboratories, GmbH, Munchen, Germany.

Millipore disc filters (0.2µm and 0.45µm pore size) and Millipore multifiltration apparatus from Millipore Corporation, U.S.A.

Sepharose 6B, Sephadex G-25 (PD 10) columns and Fast Protein Liquid Chromatography (FPLC) HiLoad 16/60 Superdex 200 column from Pharmacia Biosystems Ltd.

Ammonium sulphate, sodium carbonate (anhydrous), urea from Fisons Scientific Apparatus, Loughborough, U. K. Silicon carbide (SiC) was Grade C6-F400 from The Carborundum Co., Manchester, England.

3.2.1. Radiochemicals employed.

Radioactive orotic acid, [6-^{14}C]orotate, was purchased from ICN Flow, High Wycombe, U. K. , with specific activity of 61mCi/mmole (50μCi in 1ml).

Radioactive hypoxanthine, [8-^{14}C]hypoxanthine (specific activity of 55mCi/mmole; 50μCi as white powder) ,and [G-^{3}H]hypoxanthine (specific activity of 1.32Ci/mmole; 1mCi as white powder) were purchased from Amersham International plc.

3.2.2. Organisms used.

The bloodstream form of *T.b.brucei* was prepared and purified as described previously. The cultured procyclic cells were cultured and supplied by Marjan Kasraeian, Biochemistry Department, University of Bath. These cells were grown from a frozen stock of procyclic *T.brucei* strain EATRO-427, originally supplied by Dr. W. Gibson (Tsetse Research Centre, Langford, Bristol). Growth was at 26°C in SDM-79 [Brun, R. and Schonenberger, M. (1979) *Acta Trop.* **36**,289-292] supplemented with 10% (v/v) fetal calf serum, and cells were harvested by centrifugation at 600*g* and washed with phosphate buffered saline.

3.3.0. Methods of assay for PRPP synthetase.

Phosphate protection buffer.

KH_2PO_4	50mM, pH 7.6
$MgCl_2$	6mM
Na_2EDTA	0.1mM
Mercaptoethanol	2.5mM
PMSF	0.5mM

3.3.1. Orotate Removal Assay.

This assay was developed by Kornberg *et al.* (1955). It involves two separate steps. In the first step (step 1) enzyme activity accumulates PRPP, which is converted to orotidylic acid and uridylic acid in the second step (step 2), by an enzyme preparation from yeast which contains OPRTase and ODCase activities. The disappearance of orotate in step 2 is followed spectrophotometrically. The disappearance of 1 µmole of orotate (which is equivalent to the production of 1 µmole PRPP) from the 1ml reaction volume, corresponds to a decrease in absorbance of 3.95 at 295nm.

The assay was slightly modified as follows:

Step 1 . PRPP Formation Step.

In 1 ml final reaction volume, the reaction mixture contained:

ATP	7.5mM
R5P	4mM
$MgCl_2$	3mM (added after ATP and buffer)
KH_2PO_4	95mM
DTT	0.2mM
Trizma base buffer (pH 8.85)	100mM

and enzyme samples containing 1.25-1.45mg protein.

The final pH was adjusted to 8.85 at 37°C.

Incubation was carried out at 37°C and was stopped at 0,7.5, and 15min by heating at 100°C for 60sec.

The tubes were immediately cooled by plunging them into crushed ice and then stored overnight at -20°C. The thawed samples were centrifuged at 4000x*g* for 15min at 2°C, and from the resulting clear supernatant fluid the PRPP produced in step 1 was measured in step 2.

Step 2 . PRPP Assay.

In a 1ml quartz cuvette the reaction mixture of final volume 1ml contained:

Sodium orotate	2mM
$MgCl_2$	2mM
Tris-HCl buffer (pH 8.85 at 20°C)	20mM

4 Units of OPRTase-ODCase mixture (see materials).

0.3ml aliquot from step 1 incubation mixture.

The final pH was 8.0.

Care was taken to establish a constant absorbance reading before adding the step 1 aliquot, which was introduced with rapid mixing. The decrease in absorbance at 295nm was followed in a spectrophotometer. The reaction came to completion when there was no more change in the absorbance (about 20min).

One unit of enzyme activity is defined as the amount catalysing formation of 1μmole of PRPP per min under the conditions of the assay.

Step 2 was also used directly for enzymic assay of PRPP. In step 1 reaction mixture R5P was omitted and replaced by 165nmole of PRPP in order to check the assay. Also blanks were included in which the R5P was substituted with water.

Preparation of rat liver PRPP synthetase.

Homogenisation buffer.

Potassium phosphate	0.1M (pH 7.4)
Dithiothreitol	0.2mM

In order to standardise the Orotate Removal assay, rat liver enzyme was prepared and assayed according to the method of Baló-Banga and Weber (1984).

Wistar rats (180-200g) were stunned, decapitated, and exsanguinated; the liver was quickly removed into a beaker with homogenisation buffer (see above) which had previously been weighed and embedded in crushed ice.

A ten percent homogenate from rat liver was prepared using a blender homogeniser (15sec). The homogenate was centrifuged at 100,000x*g* for 30min at 2°C in a Beckman L5-50 preparative ultracentrifuge. To 6ml of the resulting clear supernatant, 1.4g solid ammonium sulphate was added, resulting in 40% final saturation. After 10min incubation at 0°C the precipitate formed was removed by centrifugation at 100,000x*g* for 15min at 2°C. The pellet was resuspended in 0.35ml of the homogenisation buffer and then centrifuged at 1,500x*g* for 15min in a Sorvall type RC-5 superspeed refrigerated centrifuge. From the clear supernatant 25µl samples were assayed for PRPP synthetase activity.

3.3.2. Alternative forms of the orotate removal assay.

An alternative form of this assay was the production of [^{14}C] 5'-OMP and [^{14}C] 5'-UMP from [6-^{14}C]orotate and the separation of substrate and products by TLC. The solvent utilised was n-butanol: methanol: water: ammonia (sp. gr. 0.88) ;60: 20: 20: 1, (v/v). The negatively charged products stayed nearer the origin (R_f values for OMP and UMP of 0.24 and 0.14 respectively) and the substrate, [6-^{14}C]orotate, travelled with an R_f value of about 0.37.

In this procedure, aliquots are spotted from an incubation mixture after the assay had been stopped by mixing the aliquot to 50% final concentration of ethanol and spotted on TLC plastic plates pre-coated with PEI-cellulose.

A carrier was spotted prior to the aliquot in order to aid the localisation of the substrate spot. The carrier consisted of 10µl (10mM stock) orotic acid. Suitable markers were run on either side of the test sample and the plates run for 6 hours in a pre-equilibrated (3 hours) tank. After the plates were dried, the spots were viewed under U.V. light and the position of the spots marked with a soft pencil. The plates were then cut in 0.5x1 cm^2 strips and counted with 5ml 'Optiphase' Safe scintillation fluid.

3.3.3. The coupling of PRPP synthetase to Hypoxanthine-guanine phosphoribosyltransferase.

The assay was essentially that of Roth *et al.* (1974) but with some adaptations. In principle, the activity of PRPP synthetase was measured by the PRPP-conversion of radioactive hypoxanthine (^{14}C label on a carbon atom in the purine ring, or ^{3}H label on the hydrogen of carbon atom at position 8) to radioactive 5'-IMP (label on same position as in hypoxanthine) in the presence of hypoxanthine-guanine phosphoribosyltransferase (Sigma).

TLC method.

The assay was essentially that of Roth *et al.* (1974). In this assay, the activity of PRPP synthetase was measured by the PRPP-conversion of [8-^{14}C]hypoxanthine to [^{14}C] 5'-IMP in the presence of hypoxanthine-guanine phosphoribosyltransferase. This coupling enzyme preparation introduced less protein in the assay and involved the production of one product compared to the two products of the orotate removal system.

The assay volume was 70µl.

The assay system contained:

Components.	µl added in the assay.
Buffer KH_2PO_4 (0.7M, pH 7.6)	2
$MgSO_4$ (70mM)	5
Na_2EDTA (10mM)	1
ATP (50mM)	2
R5P (50mM)	2
[G-^{3}H] Hypoxanthine solution (1.9 µmole/ml; 1.32 Ci/mmole)	4
HGPRTase (1 munit/µl)	2
Trypanosomal extract	10 or 20µl

Water up to 70µl volume..

Suitable controls were included where R5P was omitted from the assay system for all the concentrations of enzyme assayed. PRPP (1mM stock) was also included in assay systems (as 2µl/assay) without any R5P, ATP and trypanosomal enzyme preparation added, as control.

After incubating for 0, 15, 45 and 150 minutes, 10µl samples were removed at each time point and mixed with 10µl 95% ethanol and left on ice. After the addition of 5µl of hypoxanthine (2.38mM) or IMP (0.1M) carrier solution, a 20µl sample was spotted on the plastic PEI-cellulose pre-coated plastic TLC sheets with suitable markers on each side. The plates were run in the same solvent as with the alternative orotate removal assay and processed in the same manner.

The R_f values for IMP and hypoxanthine were 0.014 and 0.297 respectively. Strip blank had 30cpm and was subtracted from all strip cpm. The percentage of substrate converted to product was expressed as nmoles of PRPP produced over the time of incubation.

Detection of PRPP synthetase in bloodstream and cultured procyclic *T.brucei* cells.

The cells were resuspended in phosphate protection buffer and disrupted by sonication (see later on). The sonicate was spun at 20,000x*g* for 20 minutes at 2°C and the resulting pellet resuspended in a small volume of the same buffer and both supernatant and pellet fractions assayed by the standard method for PRPP synthetase activity described below.

DE-81 filter method. Standard method.

Materials and methods.

Stock solutions prepared.

1. Potassium buffer	0.7M, pH 7.6
2. $MgSO_4.7H_2O$	70mM
3. Na_2EDTA	10mM
4. R5P	20mM
5. ATP	50mM
6. HGPRTase	1munit/µl
7. [8-^{14}C]hypoxanthine	20µCi/ml; 55mCi/mmole

The hypoxanthine was prepared by diluting the stock 5 times with water and filtering though a DE-81 disc.

8. IMP	0.1M
9. Adenosine	4mM in 20mM phosphate buffer, pH 7.6.

The assay had a final volume of 70µl. A cocktail containing the potassium phosphate buffer, magnesium sulphate, ATP, EDTA, hypoxanthine and HGPRTase was added as 22µl. Control assays had no R5P. Water was added to make the volume to 60µl and the mixture pre-incubated for 5 minutes at 43°C in a water bath.

A suitably diluted enzyme preparation (at 0°C) was added last in 10µl to start the reaction and the incubation mixture mixed with a whirly-mixer and transfered to a second water bath equilibrated at 37°C for 10 minutes.

Reaction mixture	Final concentration in the 70µl of the assay.
Potassium phosphate, pH 7.6	20mM.
$MgSO_4$	5mM.
Na_2EDTA	0.143mM.
ATP	1.43mM.
[8-^{14}C]Hypoxanthine	7µM; ~60000cpm
HGPRTase	2munits in total.
R5P	2.87mM.

Enzyme preparation sufficient to give a linear relationship between reaction velocity and protein concentration was added last (see later on).

The reaction was stopped by placing samples in a boiling water bath for 30 seconds. The samples were chilled on ice and 10µl of the IMP stock solution added prior to filtration. The samples were filtered 12 at a time using a Millipore multifiltration unit through DEAE paper discs (Whatman DE-81) which were soaked in 4mM adenosine solution in 20mM phosphate buffer, pH 7.6. The discs were washed with 50ml water and 10ml 95% ethanol. The filters were transfered in scintillation vials and immersed in 5ml scintillation fluid (Optiphase 'Safe', LKB Ltd.). The counting time was 5 minutes for each sample.

3.3.4. ^{32}P Transfer assay.

The assay was developed by Switzer (1969) and measures the transfer of radioactivity from [γ-^{32}P]ATP to the product, PRPP, which is not adsorbable by charcoal. The labelled product is hydrolysed in acid to inorganic phosphate. Unreacted ATP and reaction product AMP are removed by adsorption by charcoal and centrifugation of the adsorbed complexes.

This assay is used in kinetic studies with highly purified enzyme. It is not suitable for use in crude extracts where there is interference from other enzymes, mainly those that involve ATPase-like activity. It is the most sensitive and rapid assay available for PRPP synthetase.

In this assay samples contain the following in a final volume of 0.5ml: 100mM potassium phosphate (pH 8.0); 10mM $MgCl_2$; 2mM ribose-5-phosphate; 1.4mM Na_2ATP; 0.1mM Na_2EDTA; 0.1mg of bovine serum albumin; [γ-^{32}P]ATP to give a specific activity of 100 to 200 cpm per nmole; and enzyme as specified.

After incubation for 30minutes at 37°C, the reaction was stopped by addition of 0.5ml of 5% (v/v) perchloric acid, and cooled on ice for at least 10min. After mixing the stopped reaction mixtures with 0.3ml of 20% (v/v) acid-washed Norit (Zimmerman, 1963) and standing in ice for 10 to 15min, 0.2ml of a 'carrier' composed of 5mg per ml of bovine serum albumin in 50mM sodium pyrophosphate, pH 7.0, was added and the substances were mixed vigorously. After centrifuging the tubes, 0.5ml of the clear supernatant fluid was removed for counting.

3.3.5. Methods of trypanosome disruption.

In order to optimise he yield and specific activity of PRPP synthetase from a given number of bloodstream forms of *T.b.brucei*, different well-known trypanosome disruption methods were attempted as described by Oduro *et al.* (1980a,b). The trypanosomes were purified from contaminating blood cells as described in chapter 2.

By sonication.

The disruption buffer used in this method was:

Tris-HCl	20mM (pH 8.0)
Sucrose	320mM
Na_2-EDTA	1mM
DTT	2mM

The cells were washed in the disruption buffer and resuspended at a density of $5x10^8$ cells/ml. The cells were sonicated at 0°C at 40 Watts for 15 sec at each time and the process repeated 4-6 times with an interval of 30 sec.

The extent of disruption was followed by microscopic examination. The sonicated cells were spun at 1,500x*g* for 10 min in a cooled centrifuge and the supernatant used for assaying for enzyme activity by the standard method.

Triton X-100.

Triton X-100 was added to the standard assay, at a final concentration of 0.1% (v/v).

By freezing and thawing in the presence of Triton X-100 (0.1%, v/v).

The cells were resuspended in phosphate protection buffer with 0.1% (v/v) Triton X-100 and disrupted by 3 cycles of freezing (liquid N_2) and thawing (37°C water bath). Homogenates were centrifuged at 20,000x*g* for 20 min at 2°C and the supernatant used as a source of enzyme activity.

By grinding with Silicon Carbide.

The disruption buffer used with this method was:

Tris-HCl	25mM (pH 7.8)
PMSF	0.5mM (added freshly)
DTT	1.0mM (added freshly)
KCl	150mM
EDTA	1.0mM
Sucrose	250mM (added freshly)

$5x10^8$ cells were spun down on a bench centrifuge (900x*g* at 2°C) and suspended in 5ml disruption buffer. The cells were ground by hand with silicon carbide (5g silicon carbide per $1x10^{10}$ cells) in an ice-cold pestle and mortar until no more live cells were visible microscopically.

The disrupted cells were collected in centrifuge tubes and washed in the disruption buffer (100x*g* for 15 min at 2°C) till no more silicon carbide remained. At each washing stage the supernatants were pooled and then centrifuged for 20 min at 900x*g* at 2°C. The large-granular fraction used in the localisation studies was prepared from the 5,000x*g* (10minutes at 2°C) supernatant. To obtain a concentrated glycosome fraction, the supernatant was spun for a further 20 min at 14,500x*g* at 2°C. The pellet contained the glycosomes and was resuspended in a small volume of disruption buffer. Addition of Triton X-100 at a final concentration of 0.1% (v/v) released the glycosomal enzymes.

3.3.6. Determination of protein concentration.

The concentration of protein in all samples was determined using the dye-binding method of Bradford (1976). The so-called 'Bio-Rad' method is a quick, one-step simple reaction that is carried out at room temperature. The details of the assay are found in the Bio-Rad Protein Assay Instruction Manual, 1984).

Bovine serum albumin was used for the construction of the standard curve which is shown in figure 3.1.

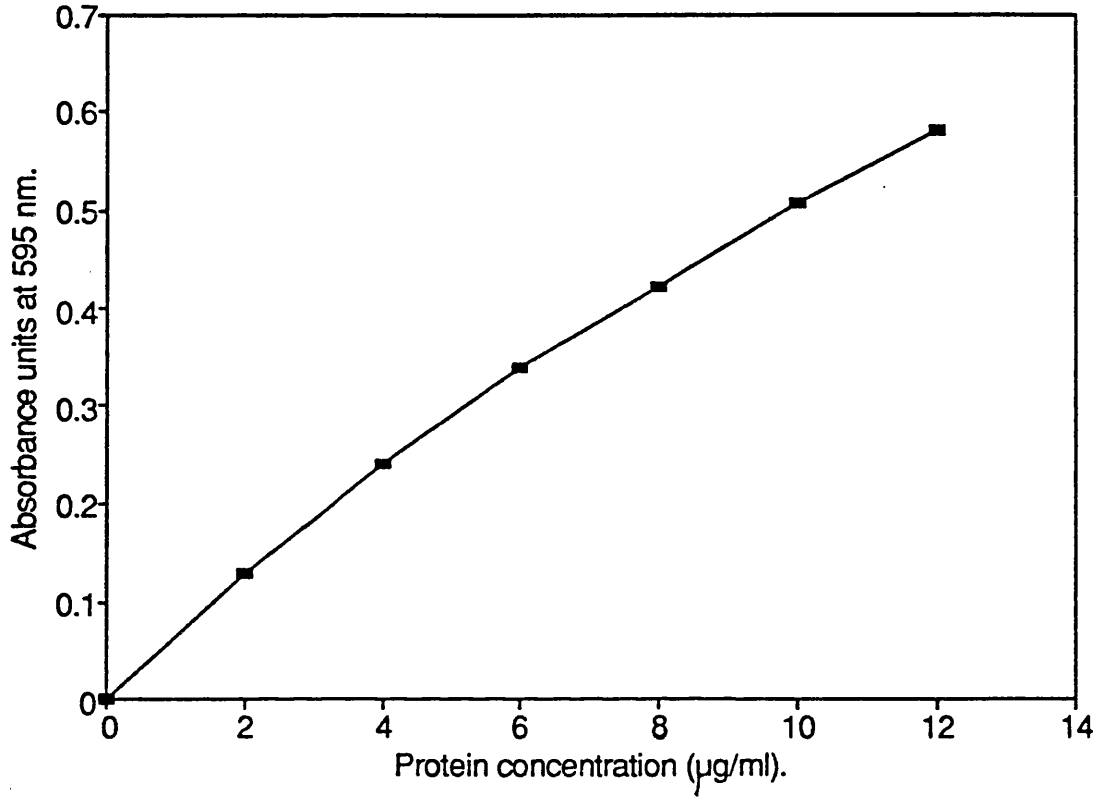


Figure 3.1. Standard callibration curve for the 'Bio-Rad' method of protein concentration estimation with Bovine serum albumin as standard in 1 ml final volume.

3.3.7. Determination of Magnesium concentration.

The magnesium concentration of the magnesium chloride stock solution was determined with Eriochrome Black T as indicator (Belcher and Nutten, 1960).

The method required the following solutions:

1. Buffer solution.

54g of ammonium chloride were dissolved in 350ml of ammonia solution (sp. gravity 0.88) and diluted to 1 L with distilled water.

2. Eriochrome Black T indicator.

0.2g of the indicator was dissolved in 15ml of triethanolamine and 5ml of ethanol. (This solution will remain stable for about 1 month).

3. 0.1 M EDTA solution.

This was prepared by dissolving, 37.22 g of disodium EDTA (dihydrate) in 1L distilled water.

4. Test solution 1 M $MgCl_2$.

This was prepared by dissolving approximately 203.31 g $MgCl_2.6H_2O$ into 1L distilled water.

The method involved the titration of 550µl of the $MgCl_2$ solution with 50µl of buffer solution and 15 drops of 10x diluted indicator solution against the 0.1 M EDTA solution in a 10ml biuret pipette until the wine-red colour changed to blue. Care was taken to approach the end-point slowly.

To neutralise from red-wine to clear blue solution, 4.85ml, 5.02ml, 5.1ml, 5.02ml and 5.14ml of 0.1 M EDTA were required. On average, 5.028ml was used in the calculation of the accurate concentration of Mg^{2+} in the $MgCl_2$ solution as follows:

$$5.028ml \times 0.1\ M = 0.55ml \times [Mg^{2+}]\ M$$

$[Mg^{2+}] = 0.914$ M solution instead of 1 M. The solution was divided in aliquots which were stored frozen at -20°C until required.

3.3.8. The partial purification of PRPP synthetase from *T.brucei.*

Ammonium sulphate precipitation.

A 20,000x*g* supernatant (20K sn) was subjected to sequential ammonium sulphate precipitation. A suitable amount of finely ground ammonium sulphate according to Dawson *et al.* (1969) was added slowly to the sample to bring it to the first 'cut'. The addition was carried out as the sample was continuously being stirred on ice, and after a further 15 minutes the sample was spun at 20Kx*g* for 20 minutes in an MSE-18 High Speed centrifuge. The pellet was resuspended in a suitable small volume of phosphate protection buffer and 'desalted' on a small Pharmacia Sephadex G-25 gel filtration column, eluted with phosphate protection buffer. The eluate was then assayed for PRPP synthetase activity and protein concentration.

The volume of the decanted supernatant was measured accurately and sufficient solid ammonium sulphate added to bring the ammonium sulphate concentration to that of the next 'cut' and the procedure repeated as above until the highest ammonium sulphate concentration had been reached.

Streptomycin sulphate precipitation.

Streptomycin sulphate, 10% (w/v), was prepared freshly for each purification in water and chilled on ice. To the enzyme sample 0.1 volume of the streptomycin sulphate solution was added and the sample stirred for 15 minutes, centrifuged and the supernatant and resuspended pellet assayed for enzyme activity using the standard assay. A small volume of phosphate protection buffer was used for resuspending the pellet.

A further 0.2 volumes of streptomycin sulphate solution were added to the carefully measured 1% supernatant, and the procedure repeated.

Heat precipitation.

A 5ml sample of the 20-40% ammonium sulphate resuspended pellet (20-40% AS P) was divided into five fractions of equal volume (1ml) and placed in glass tubes each containing a small stirring bar. Each tube was placed into a pre-heated water bath of either 50° or 55°C for 5, 10 or 15 minutes with continuous stirring with an immersible magnetic stirrer. After the desired heating time the tubes were chilled by immersion in ice-cold water and allowed to cool down prior to centrifugation at 20,000x*g* for 20 minutes at 2°C in a bench centrifuge. The supernatants were assayed and the specific activity and percentage recovery of units of PRPP synthetase from each system determined.

Coupling of Cibacron Blue F3G-A to Sepharose 6B.

To 150ml Sepharose 6B gel, 2.5g of Na_2CO_3 in 50ml water and 1g Cibacron Blue F3G-A, and in 50ml water were added. After careful mixing, the suspension was incubated for 40 hours at 45°C with occasional stirring. The non-coupled dye was removed by extensive washing with distilled water on a Buechner filter. The gel was stored at 4°C in the presence of 1g sodium azide (NaN_3).

Two column volumes were used for washing and equilibrating the column prior its use. The column was washed with 1M NaCl and 6M urea and equilibrated with phosphate protection buffer.

Routine partial purification procedure.

The trypanosome cells were routinely disrupted by three cycles of freezing and thawing and the extract allowed to incubate with Triton X-100 for 15 minutes on ice. The extract was then spun at 20,000x*g* for 20 minutes at 2°C in a bench centrifuge and the supernatant brought up to 20% ammonium sulphate concentration. The 20% ammonium sulphate supernatant was then brought to 40% concentration of ammonium sulphate and the pellet resuspended in a small volume of phosphate protection buffer with PMSF freshly added to it.

The sample was then heated for 5 minutes at 55°C in a water bath with continual stirring. The final supernatant was dialysed overnight against phosphate protection buffer containing 20% (v/v) glycerol to get rid of the ammonium sulphate and to make the sample contain glycerol for extra stability. The sample was concentrated by polyethylene glycol (20,000) and stored at 4°C until required. The enzyme preparation was used up to a week since the day of preparation.

3.3.9. Methods to test the validity of the coupled-enzyme assay.

To test that the observed rates of production of [^{14}C] 5'IMP truly represent the rate of the PRPP synthetase-catalysed reaction, the variation in measured overall reaction rate with concentration of coupling enzyme was determined.

When conditions were found under which the rate of the overall reaction was proportional to the rate of the PRPP synthetase reaction, this was further checked by studying the initial rate as a function of PRPP concentration (protein concentration of a crude preparation) at the concentration of [^{14}C]Hypoxanthine in the assay and the number of units of coupling enzyme chosen.

Under steady-state conditions, the rate of the overall reaction, that is the rate of IMP formed per unit time, should be dependent on the rate of the PRPP synthetase reaction. Doubling the PRPP synthetase concentration, should double the cpm on the filter (*i.e.* the product of HGPRTase) if HGPRTase is in excess. Because in the coupled assay, the rate of HGPRTase depends on the rate of PRPP synthetase, all substrate from PRPP synthetase should be accessible to HGPRTase (or if not all, a constant steady-state concentration).

3.3.10. Localisation of PRPP synthetase in the bloodstream form of *Trypanosoma brucei*.

The subcellular distribution of PRPP synthetase in the bloodstream form of *Trypanosoma brucei* was investigated using fractions obtained from isopycnic

sucrose gradient centrifugation of post-large-granule extract. The fractions were kindly supplied and analysed by Dr.F.R.Opperdoes' research group in the International Institute of Cellular and Molecular Pathology, Brussels. The analysis of each fraction involved the assay of a number of marker enzymes whose subcellular localisation has already been accomplished.

Materials and methods.

Growth, isolation and purification of the bloodstream trypomastigote forms of *T.brucei* stock 427 were conducted as described by Opperdoes *et al.* (1976a).

Organisms were disrupted by grinding with silicon carbide according to Toner and Weber (1972), using 0.25M sucrose, 25mM Tris-HCl and 1mM EDTA (pH 7.2) for homogenisation. The homogenates were fractionated into a nuclear fraction, a large-granule (mitochondria and microbody enriched) fraction, a small-granule fraction and the final supernatant as described by Opperdoes *et al.* (1977a).

For isopycnic centrifugation 7ml of a large-granule fraction (65.3 mg protein) was layered on top of a linear density gradient from 0.4-2.0 M sucrose containing 1mM EDTA (sodium) and 25mM Tris-HCl buffer (pH 7.4) which was prepared with a Beckman density gradient former and layered on top of a 2.5 M sucrose cushion. The gradient was centrifuged in a E-40 automatic zonal rotor in a Beckman L2-65B ultracentrifuge at 30,000rpm for 17 hours at 6°C. The gradient was pumped out through a fine bore metal tube from the bottom of the centrifuge tube (Opperdoes *et al.*, 1977b) and stored in liquid nitrogen (Opperdoes and Borst, 1977).

The enzymatic analyses of the protein and marker enzymes in the fractions were carried out by the group in Brussels using the following published methods: alanine aminotransferase (Reitman and Frankel, 1957) ; α-mannosidase (Peters *et al.*, 1972) ; α-glucosidase (Barrett and Heath, 1977) ; acid phosphatase (Opperdoes *et al.*, 1977a) ; hexokinase (Bergmeyer, 1974) ; NADP-linked

isocitrate dehydrogenase (Ochoa, 1955) ; 3'-nucleotidase (Gottlieb and Dwyer, 1983) and protein by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

All 14 fractions were kindly transported from Brussels by Simon Jackman. They were each diluted 8-10 times in the following buffer,

Potassium phosphate	50mM, pH 7.6
Na_2EDTA	0.1mM
$MgCl_2$	6mM
Mercaptoethanol	2.5mM
PMSF	0.5mM
Triton X-100	0.25% (v/v)

The activity of PRPP synthetase was measured using the standard assay (see methods section) on 10µl of the diluted sample of each of the 13 fractions supplied, including the homogenate fraction 14, representing the supernatant from the 5000x*g* large-granular fraction which was placed on the gradient. The PRPP synthetase activity of each fraction was measured in duplicate samples using three different protein concentrations in the assay. The mean average taken as activity/ml, munits PRPP synthetase/ml, was expressed as a percentage of the activity of the homogenate which was 100%.

3.3.11. Gel filtration of PRPP synthetase from *T.brucei.*

Method.

Gel filtration was carried out with a prepacked HiLoad 16/60 Superdex 200 Pharmacia fast protein liquid chromatography column using a mechanised Pharmacia fast protein liquid chromatography system. The column was 60cm high and 16mm in diameter and was equilibrated with 50mM potassium phosphate, pH 7.6; 6mM magnesium chloride, 1mM EDTA (disodium salt) and 2.5mM mercaptoethanol. The void volume of the column was determined with Dextran blue 2000 and calibrated with standard proteins of known molecular

weight *viz.* carbonic anhydrase (29,000); bovine serum albumin (66,000); alcohol dehydrogenase (150,000); β-amylase (200,000); apoferritin (443,000) and thyroglobulin (669,000). MilliQ grade water was used throughout buffer preparation and all buffers and samples were pre-filtered through Millipore (0.2μm) filters. The column flow rate was 48ml/hour and 1ml fractions collected. The runs were carried out at room temperature. The activity of PRPP in the fractions was assayed in 10μl aliquots using the standard method.

Gel filtration was carried out on two enzyme preparations at different stages of purification.

Gel filtration of partially purified enzyme preparation.

The routine purification procedure was carried out and the dialysed 55°C heat fraction was concentrated to a 3ml volume with polyethylene glycol (20,000) and 200μl applied to the Superdex 200 column. The column was eluted in the equilibration buffer and the active fractions pooled. The pooled fractions were dialysed against water, concentrated with polyethylene glycol and gel electrophoresed.

Gel filtration of crude homogenate of *T.brucei.*

The crude homogenate was prepared by the grinding method of trypanosome disruption (see method section). The column was equilibrated in the disruption buffer, 25mM Tris-HCl (pH 7.2), 1mM EDTA (disodium salt) and sucrose 0.25M. The 5,000x*g* (10minutes at 2°C) supernatant was filtered through a 0.45 m Millipore filter which was pre-rinsed in the disruption buffer and a 2ml sample applied onto the Superdex column. The column was eluted with disruption buffer.

3.4.0. Results.

3.4.1. Drawbacks of the Orotate Removal Assay.

The assay is insensitive for activities below 2nmoles PRPP formed/min. It requires the use of a sensitive spectrophotometer such as a Cary Model 118 CX recording spectrophotometer which was not available for the present work. The amount of protein in the assay was very high (>8mg) and the contribution at 295nm of the absorbance of the protein (>2.6 absorbance units) in the OPRTase-ODCase preparation meant that small absorbance changes were impossible to detect or measure accurately due to the insensitive absorbance scale of the Unicam recording spectrophotometer employed.

This method failed to detect PRPP synthetase activity in trypanosomal extracts but was adequate for detection and quantitation of rat liver PRPP synthetase activity. This was measured in order to investigate whether the lack of activity with trypanosomal extracts was due to the assay failure or otherwise. The rat liver PRPP synthetase activity was estimated to be ~120-185nmoles/hr/mg protein. This result was slightly higher than that recorded in the Balo-Banga and Weber (1984) paper of 80-125nmoles/hr/mg protein.

When trypanosome extract was assayed in the presence of rat liver PRPP synthetase preparation, there was no difference to the activity of the same sample of rat liver enzyme preparation assayed on its own. This showed that there was no inhibitors of the mixed enzymes in step 2 of the assay in the trypanosome crude preparation. The spectrophotometric orotate removal assay was therefore abandoned and more sensitive radioactive assays employed instead.

3.4.2. Alternative form of Orotate removal assay.

Using this method it was possible to show that the trypanosome crude extract contained PRPP synthetase activity, due to the increasing number of counts accumulating at the product spots with increasing reaction time. A typical result of progressive accumulation of product with increasing incubation time is shown in Figure 3.2.

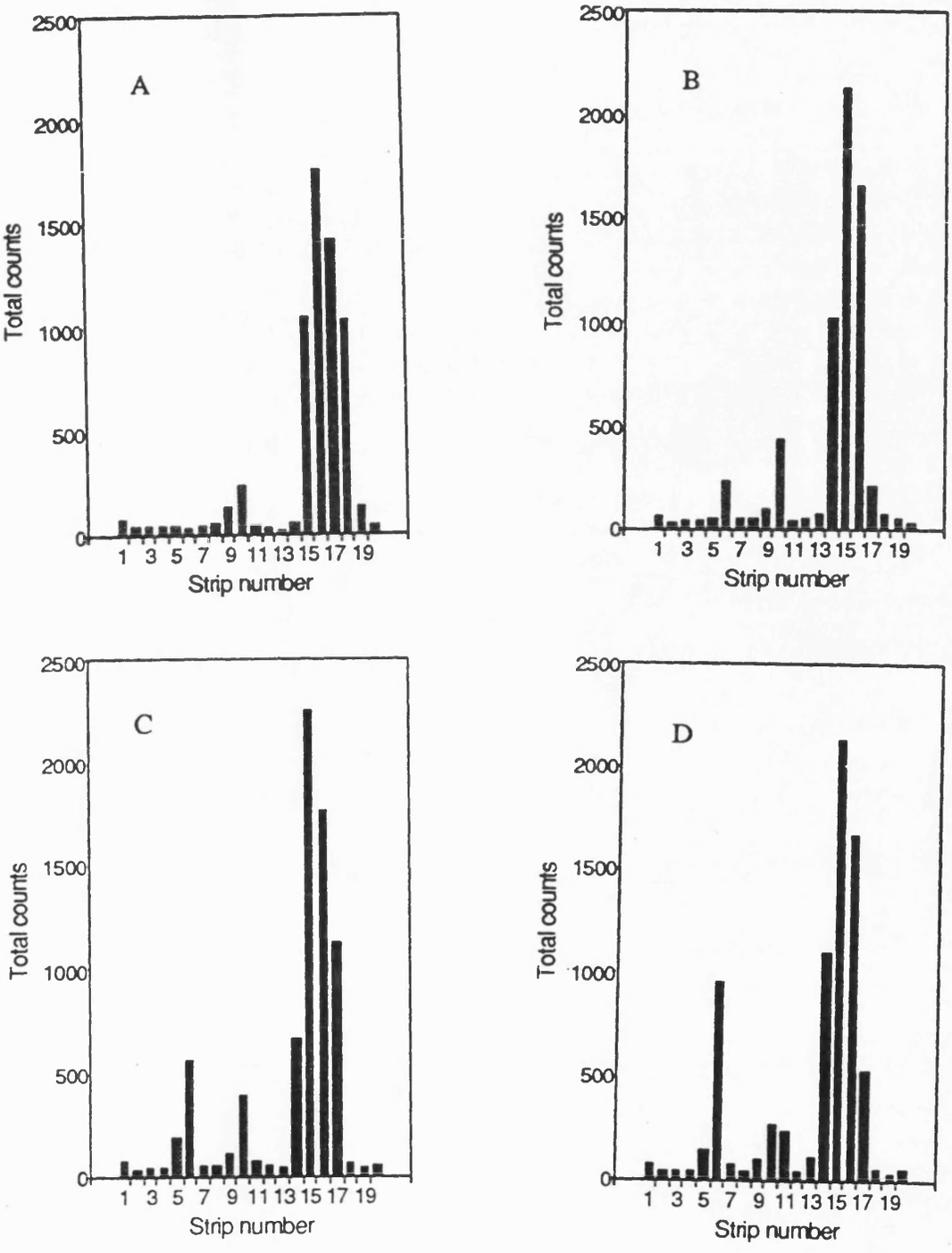


Figure 3.2. Orotate removal assay. Accumulation of product with increasing time of incubation. A, B, C, and D correspond to time 0, 20, 60, and 180 minutes respectively. Strips 5-7, 9-11 and 14-18 correspond to [^{14}C] 5'-UMP, [^{14}C] 5'-OMP and [^{14}C] orotate respectively. The amount of PRPP in the assay was 7 nmoles in total in the 60 µl final assay volume.

Up to 20 strips were used for every assay which resulted in a great number of vials and counter time.

Although the separation of the substrate (orotic acid) and products (UMP and OMP) was adequate, the method was discontinued as it proved impractical. The alternative method tried employed the coupling of PRPP synthetase to HGPRTase.

3.4.3. The coupling of PRPP synthetase to Hypoxanthine-guanine phosphoribosyltransferase.

TLC method.

The results of a typical experiment are shown in Table 3.1. The counts present in the substrate (hypoxanthine) and product (IMP) strips of the TLC plate were expressed as percentages of the total counts of the assay applied to the TLC plate and marked as S and P respectively. Control assays were carried out with ommission of R5P from the assay. It can be seen that in all the assays and controls there was a progressive accumulation of radioactivity for P and a respective decrease in counts for S. There was double the amount of product formed at each incubation time as the amount of trypanosomal extract was doubled indicating that the overall reaction rate was proportional to the amount of PRPP synthetase present in the assay. The result obtained with the control assays indicated the presence of one or more other contaminating enzymic activity able to phosphorylate hypoxanthine without R5P and PRPP requirement.

DE-81 filter method.

. Under assay conditions, over 95% of IMP was retained on the disc, while the hypoxanthine was not retained at all. The background was 0.3% of the total counts filtered through.

Table 3.1. TLC method of HGPRTase-coupled assay.

Assay		Time of incubation (minutes)			
		0	15	45	150
sn C1 + R5P	P	0.686	1.168	1.501	2.466
	S	97.630	96.229	97.630	95.123
sn C1 - R5P	P	0.778	1.041	1.174	1.390
	S	97.679	97.311	97.457	96.773
sn C2 + R5P	P	0.633	1.733	2.769	4.628
	S	97.126	95.979	95.111	93.573
sn C2 - R5P	P	0.973	1.453	2.175	2.382
	S	97.489	96.825	96.781	95.696

P and S represent the percentage of total counts present in each TLC strip as product ([^{3}H]IMP) and substrate ([^{3}H]hypoxanthine) respectively. C1 and C2 are 10 and 20µl of trypanosomal 20K sn preparation respectively. Control assays were carried out in the absence of R5P in the assay.

3.4.4. Different methods of trypanosome disruption.

From the results in Table 3.2 it was clear that there was very little difference in the yield and specific activity of the enzyme obtained by the different disruption methods. The disruption of the cells was routinely achieved by the freezing and thawing method in the presence of Triton X-100 at 0.1% (v/v) final concentration.

3.4.5. The partial purification of PRPP synthetase from *T.b.brucei.*

The different stages in the development of the partial purification procedure for trypanosomal PRPP synthetase are presented in Tables 3.3-3.5 inclusive. The routine partial purification protocol was developed and is presented in Table 3.6. On average the recovery of enzyme units in the dialysed enzyme preparation was 56% and the purification ford achieved was about 73x that of the crude extract.

3.4.6. Removal of an endogeneous inhibitor from the crude extract.

It was observed that there was a greater yield than 100% on some steps in the purification procedure such as after the supernatant of the 0-40% ammonium sulphate 'cut' was removed. A similar increase in unit numbers recovered was achieved by dialysis of the 20K sn obtained after disruption of the cells by freezing and thawing and also after passing the 20K sn fraction through a Pharmacia Sephadex G-25 'desalting' column (Table 3.7). From those treatments it is possible to suggest that there is an endogeneous inhibitor present in the crude extract that has a small enough molecular weight to be retained by the gel filtration column.

Table 3.2. Results from different methods of trypanosome disruption.

The results were expressed as values per 10^9 cells.

Method of trypanosome disruption	Volume (ml)	Protein (mg)	Total activity (mUnits)	Specific activity (mUnits/mg protein)
Sonication	0.976	4.488	0.462	0.103
Triton X-100	1.126	2.139	0.326	0.153
Freezing and thawing with Triton X-100	0.331	2.120	0.371	0.175
Grinding (SiC)	1.397	2.164	0.229	0.106

Where Triton X-100 is included it was always at a final concentration of 0.1% (v/v). The activity of PRPP synthetase is expressed in mUnits *i.e.* nmoles PRPP formed/minute using the standard assay.

Table 3.3. Ammonium sulphate precipitation.

System	Volume (ml)	Protein (mg)	Activity (mUnits)	Specific activity (mU/mg)	Recovery (%)	Purification fold (x)
Crude	4.66	47.53	4.52	0.095	100.00	1.00
20K sn	4.00	32.80	4.02	0.123	88.94	1.29
20K P	0.80	14.24	0.22	0.015	4.87	0.16
Ammonium sulphate resuspended pellets						
0-15 %	3.50	10.250	0.33	0.032	7.19	0.34
15-25 %	3.40	8.630	4.79	0.555	105.97	5.84
25-35 %	3.60	4.360	3.82	0.876	84.51	9.22
35-45 %	3.50	3.900	0.82	0.209	18.03	2.20
45-55 %	3.40	0.386	0.02	0.041	0.35	0.43

These are the results of the initial 'cuts' of ammonium sulphate (AS) tried. The standard assay was carried out on suitably diluted samples in phosphate protection buffer. Each pellet had been passed through a Sephadex G-25 'desalting' column prior to assaying.

Table 3.4. Ammonium sulphate and streptomycin sulphate precipitation.

System	Volume (ml)	Protein (mg)	Activity (mUnits)	Specific activity (mU/mg)	Recovery (%)	Purification fold (x)
Crude	7.03	35.62	3.74	0.105	100.00	1.00
20K sn	6.20	21.55	2.63	0.122	70.30	1.16
Ammonium sulphate						
0-20 % sn	6.05	20.39	1.68	0.082	44.900	0.78
0-20 % P	3.00	0.19	0.01	0.053	0.270	0.50
20-40 % sn	6.00	20.20	0.07	0.003	1.870	0.03
20-40 % P	4.17	4.59	4.88	1.063	130.480	10.12
Streptomycin sulphate						
0-1% sn	5.16	2.45	3.03	1.237	81.02	11.78
0-1% P	1.04	0.34	0.10	0.294	2.67	2.80
1-3% sn	3.50	0.73	2.61	3.575	69.79	34.05
1-3% P	0.38	0.02	0.01	0.500	0.27	4.76

The standard assay was carried out on 10µl aliquotes of 10-fold diluted samples in phosphate protection buffer.

Table 3.5. Heat precipitation of the 20-40% ammonium sulphate pellet.

System	Volume (ml)	Protein (mg)	Activity (mUnits)	Specific activity (mU/mg)	Recovery (%)	Purification fold (x)
20-40 % AS P	6.1	5.400	3.11	0.576	158.00	8.06
aliquot of AS P	1.0	0.885	0.51	0.576	100.00	1.00
Supernatants obtained after 1.0 ml aliquot was heat treated at 50°C						
5 min	1.0	0.325	0.868	2.672	170.3	4.64
10 min	1.0	0.158	0.504	3.191	98.9	5.54
15 min	1.0	0.120	0.483	4.023	94.7	6.98
Supernatants obtained after 1.0 ml aliquot was heat treated at 55°C						
5 min	1.0	0.161	0.708	4.398	138.8	7.64
10 min	1.0	0.150	0.324	2.160	63.5	3.75

PMSF was freshly added to 0.5mM final concentration in the phosphate protection buffer used in the resuspension of the ammonium sulphate pellet (AS P) . One ml of that preparation was heat treated and the denatured protein removed by centrifugation at 4°C in a bench centrifuge. The activity of PRPP synthetase is expressed as mUnits which is the amount of enzyme required to produce 1nmole of PRPP per minute at 37°C in the standard assay used.

Table 3.6. Routine parial purification procedure for trypanosomal PRPP synthetase.

System	Volume (ml)	Protein (mg)	Activity (mUnits)	Specific activity (mU/mg)	Recovery (%)	Purification fold (x)
crude	16.70	76.82	8.61	0.112	100.00	1.00
20K sn	14.47	49.82	6.78	0.136	78.75	1.21
20-40 % AS P	6.70	6.20	10.41	1.679	120.90	14.99
5 min sn at 55°C	6.00	1.14	7.00	6.140	81.30	54.82
dialysed enzyme.	1.62	0.59	4.84	8.203	56.21	73.24

The dialysed enzyme preparation represents the product of overnight dialysis against 500 volumes of phosphate protection buffer with 20% (v/v) glycerol and concentrated with polyethylene glycol (20,000). The samples were diluted 10 fold in phosphate protection buffer and 10µl aliquots assayed by the standard assay.

Table 3.7. Removal of an endogeneous inhibitor and activation of enzyme extract by passing through a G-25 'desalting' column.

System	Volume (ml)	Protein (mg)	Activity (mUnits)	Specific activity (mU/mg)	Recovery (%)	Purification fold (x)
Crude	4.44	34.74	3.93	0.113	100.00	1.00
20K sn	3.90	24.96	3.81	0.153	96.99	1.35
20K sn through G-25	7.38	20.57	7.37	0.358	187.81	3.17

The crude preparation was obtained by disrupting the cells with 3 cycles of freezing and thawing in liquid nitrogen and 37°C respectively. The cell debris was spun down at 20K*xg* at 4°C for 20 minutes to give the 20K sn fraction. This was passed through a Pharmacia G-25 column. All the samples were assayed using the standard assay.

3.4.7. Cibachron Blue-Ligand chromatography.

Elution of PRPP synthetase with 6mM ATP at pH 7.6.

The result is shown in figure 3.3. This procedure gave a recovery of PRPP synthetase units of 15% and of protein of 75%. The purification fold of fraction 26 was 27 fold.

This method was not used further due to the low enzyme recovery.

Elution of PRPP synthetase with 2mM ATP at different pH.

The results of this experiment are presented in Table 3.8. The system of pH 6.5 gave the highest percentage recovery of units and purification fold but not of any great significance so dye-ligand chromatography was abandoned as a purification step for trypanosomal PRPP synthetase.

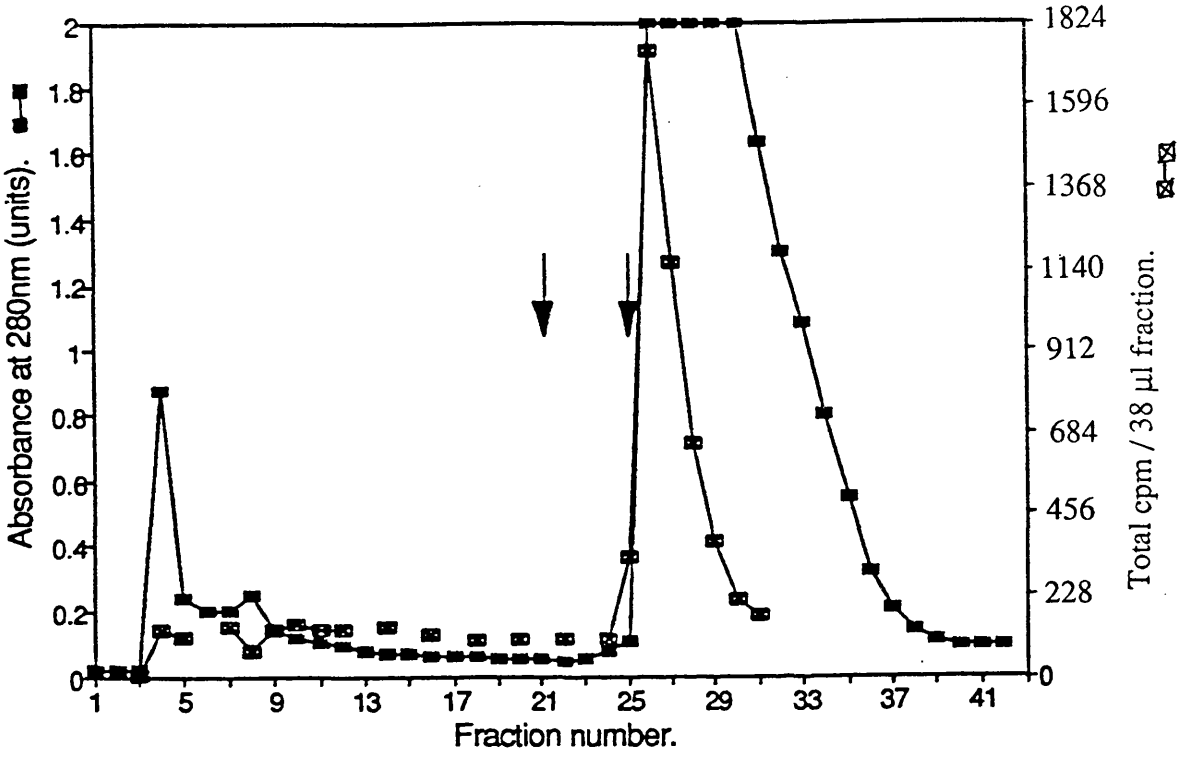


Figure 3.3. Elution of PRPP synthetase from Cibacron-blue affinity chromatography column with 6mM ATP at pH 7.6. A Cibacron Blue-Sepharose column (20 x 1.6 cm) was equilibrated with phosphate protection buffer, pH 7.6 and 7ml of 1% sn from streptomycin sulphate precipitation (2.417 mg protein of 0.307 mUnits/mg specific activity) were applied; the column was eluted with 132 µmoles of ATP in phosphate protection buffer. The ATP was applied between the two arrows. A flow rate of 0.62 ml/min was used and fractions collected every 10 minutes. The standard assay was used with 38µl samples of each fraction.

Table 3.8. Elution of PRPP synthetase from Cibacron-blue affinity chromatography column at different pH phosphate buffers.

System	Volume (ml)	Protein (mg)	Activity (mUnits)	Specific activity (mU/mg)	Recovery (%)	Purification fold (x)
20K sn loaded	1.17	12.40	1.58	0.127	100.00	1.00
pH 6.5						
W	11.13	8.510	0.38	0.044	23.81	0.35
E	6.05	0.256	0.12	0.481	7.81	3.79
pH 7.0						
W	11.02	7.600	0.44	0.058	28.00	0.46
E	5.80	0.260	0.06	0.239	3.94	1.88
pH 7.5						
W	11.02	8.375	0.50	0.060	31.94	0.47
E	5.95	0.402	0.06	0.157	4.00	1.24
pH 8.0						
W	11.02	8.10	0.66	0.082	42.16	0.65
E	5.95	0.39	0.06	0.144	3.56	1.13

Where W and E correspond to washing and elution fractions respectively.

3.4.8. Detection of PRPPsynthetase in bloodstream and cultured procyclic *T.b.brucei* cells.

In Table 3.9 the content of PRPP synthetase expressed in munits from bloodstream forms (BS) vas found to be considerably lower than that of the same number of cultured procyclic (CP) forms. The difference might reflect genuine differences in the PRPP requirements of the CP forms for growth and replication. The estimation for the CP forms was carried out only once so it was not possible to derive much information from it. From the raw data (counts per minute) it was immediately obvious how the observed difference arose since the CP crude extract gave a very low blank value compared to the consistently high value of the BS form crude extract.

The attempted estimation of the sum total of PRPP and R5P concentrations may not have been accurate due to the lack of boiling the enzyme extract to kill the PRPP synthetase. This was not done because PRPP is very easily destroyed by extensive heating. From Table 3.9, it appeared that the sum content of PRPP and R5P concentrations in the two forms was very similar.

3.4.9. Validity of the coupled-enzyme assay.

In determinations of catalytic activity, the task of the coupling (indicator) reaction is to indicate at any time point the amount of reaction product of the primary reaction. The rate of the coupled overall reaction is followed on the basis of the increase of the reaction product of the indicator reaction with time (Bergmeyer, 1983).

In the coupled reaction:

$$S_1 + S_2 \xrightarrow{\text{Eprim.}} P_1 + P_2$$

$$P_1 + S_3 \xrightarrow{\text{Eind.}} P_3 + P_4$$

Table 3.9. PRPP synthetase and the sum of PRPP and R5P in the bloodstream and cultured procyclic *T.b.brucei* cells.

Form of *T.b.brucei*	[PRPP + R5P] content (nmoles/10^9 cells)	PRPP synthetase content (mUnits/10^9 cells)
BS form	3.375 ± 1.26	0.368 ± 0.193
CP form	2.89	1.593

BS (bloodstream form of *T.b.brucei*)
CP (cultured procyclic form of *T.b.brucei*).

The [PRPP + R5P] content was obtained from the standard assay carried out in the absence of added
R5P. Any activity observed would be due to PRPP or/and R5P already present in the extract.

The activity of PRPP synthetase is expressed as mUnits of enzyme which is the amount of enzyme required to produce 1nmole of PRPP per minute in the standard assay.

the effective rate of the indicator reaction in the simplest case is given by the formula:

$$(v_{eff.})_{ind.} = \frac{V_{ind.}}{1 + \frac{(Km)_{P1}}{[P_1]} + \frac{(Km)_{S3}}{[S_3]}}$$

In the steady-state, (veff.)ind. = Vprim. and $Q' = (Km)_{P1}/[P_1]$ is a constant. From this it follows that:

$$(v_{eff.})_{ind.} = \frac{V_{ind.} \times [S_3]}{(1 + Q) \times [S_3] + (Km)_{S3}} = V_{prim.}$$

This is the equation of a one-substrate reaction. If it is assumed that, in addition, $(Km)_{P1}/[P_1] = Q'$ must have a particular value, then $(1 + Q') \times [S_3] >> (Km)_{S3}$, the equation becomes:

$$(v_{eff.})_{ind.} = V_{prim.} = \frac{1}{(1 + Q')} \times V_{ind.}$$

or

$$V_{ind.} = (1 + Q') \times V_{prim.}$$

The condition $(1 + Q') \times [S_3] >> (Km)_{S3}$ should always be satisfied.

In the present PRPP synthetase (Eprim.) standard assay,S1 can be $MgATP^{2-}$; S_2 can be R5P; P_1 can be MgPRPP; P_2 can be AMP; Eind. can be HGPRTase; S_3 can be [^{14}C]Hypoxanthine; P_3 can be [^{14}C] 5'IMP and P_4 can be PPi.

The Km of the yeast HGPRTase for PRPP is less than 4×10^{-5} M, as described by Kornberg, Lieberman, and Simms (1955).

3.4.10. Rate of overall reaction at two [^{14}C]Hypoxanthine concentrations in the assay.

From the results in Table 3.10, there is very little difference in the rate of the overall reaction at the two final [^{14}C]Hypoxanthine concentrations in the assay. The [^{14}C]Hypoxanthine concentration used in subsequent standard assays was about 7μM.

3.4.11. Overall rate of reaction with increasing amounts of HGPRTase units in the assay.

From the results in Figure 3.4, it was apparent that the chosen 2munits of HGPRTase activity in the standard assay was adequate for ensuring that the PRPP formed in the PRPP synthetase reaction was converted to IMP by the coupling enzyme HGPRTase.

3.4.12. Percentage conversion of hypoxanthine to IMP as a function of protein concentration in the assay.

The concentration of PRPP synthetase is proportional to the protein concentration in the preparation used in the assay (Figure 3.5).

The standard assay was valid for up to 8% conversion of hypoxanthine to IMP under the conditions of the assay.

3.4.13. The percentage of hypoxanthine converted to product (IMP) as a function of assay time.

The reaction is linear with up to at least 15 minutes of incubation at 37°C. The chosen reaction time for all subsequent standard assays was 10 minutes at 37°C (Figure 3.6).

This allowed the assaying of 22 separate assays in each run since the assays were carried out in duplicate, the number of assays was up to 5 including the standard assay which always accompanied the different investigations. Since

Table 3.10. Rate of overall reaction at two [^{14}C]Hypoxanthine concentrations in the assay.

Experimental conditions as in Figure 3.4.

Concentration of [^{14}C]hypoxanthine in the assay (μM)	cpm [^{14}C] 5'IMP formed in 10 minutes with 1.7 μg protein in assay	cpm [^{14}C] 5'IMP formed in 10 minutes with 3.4 μg protein in assay
4.16	3523.6, 3144.8	5526.5, 5426.0
12.47	3558.7, 3539.2	6650.8, 6427.2

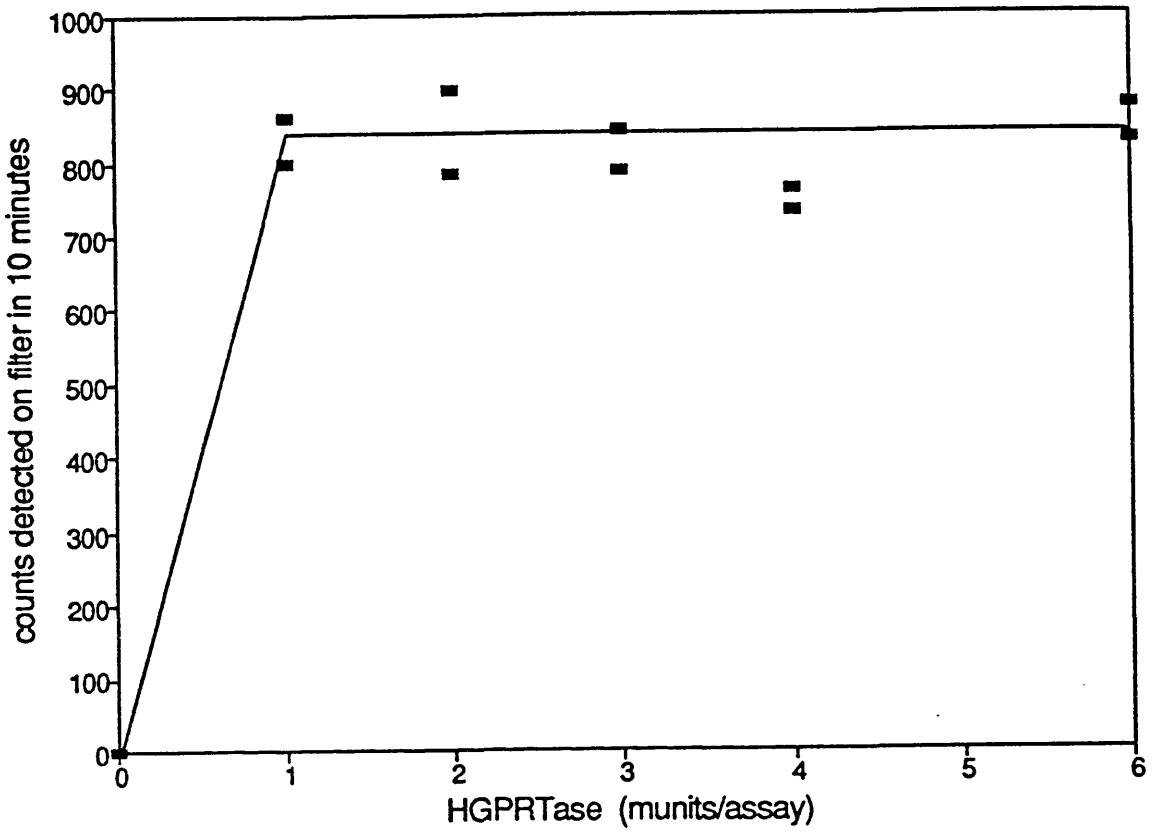


Figure 3.4. Overall reaction rate with increasing amounts of HGPRTase units in the assay.

The standard assay was carried out using 1munit, 2munits, 3munits, 4munits and 6munits of HGPRTase per 70µl assay. The volume of the assay was kept at 70µl. One munit of HGPRTase is defined as the amount of enzyme that catalyses the formation of one nmole of GMP per minute under the defined conditions. PRPP synthetase concentration was kept constant at 0.6 µg protein/assay (specific activity of 1.14 mUnits/mg protein). The reaction was carried out at 37°C for 10 minutes. The [^{14}C] Hypoxanthine concentration in the assay was 5.7 µM (48,738 cpm=100%). The arrow indicates the number of HGPRTase munits used in subsequent experiments.

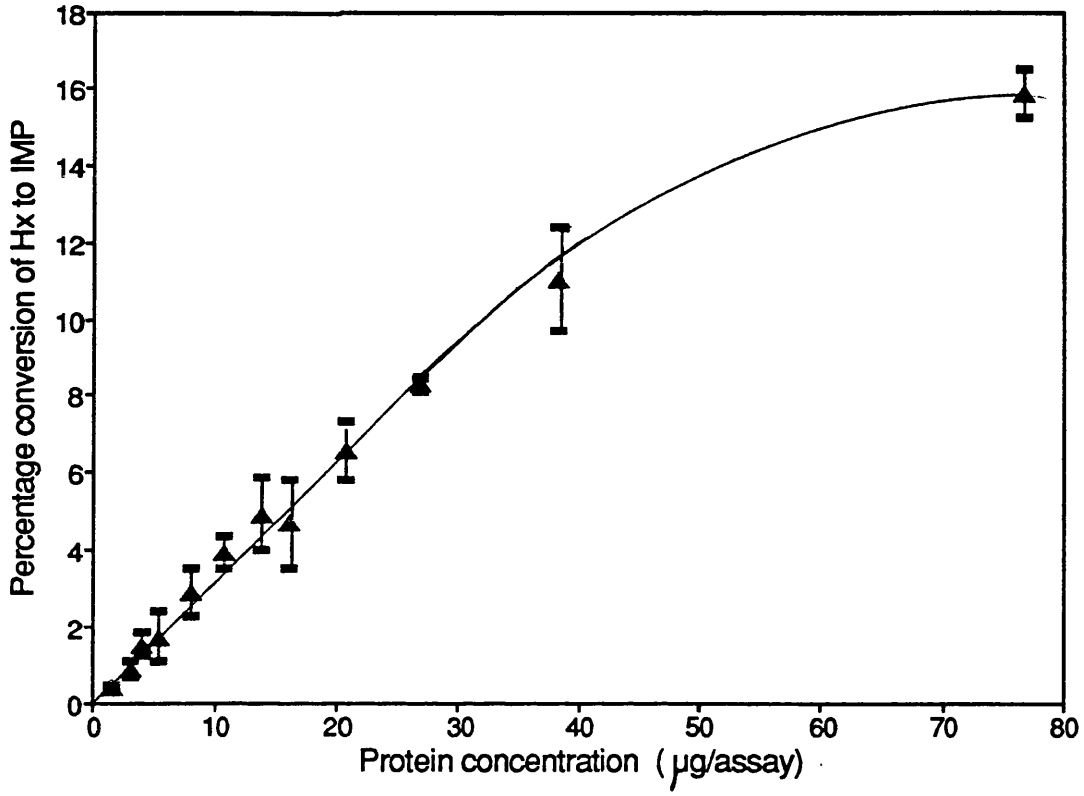


Figure 3.5. Percentage conversion of hypoxanthine to IMP as a function of protein concentration in the assay.

The assay was allowed to take place over 10 minutes at 37°C. 100% represents all the [^{14}C]Hypoxanthine supplied in the 70µl assay (56089.2 cpm). A crude preparation was used which was first passed through a Sephadex G-25 column (specific activity of PRPP synthetase 0.605 mUnits/mg protein). The concentration of HGPRTase was kept constant throughout at 2mUnits in the assay. The bars represent the mean and the error bars the standard error of the mean for 4 separate experiments.

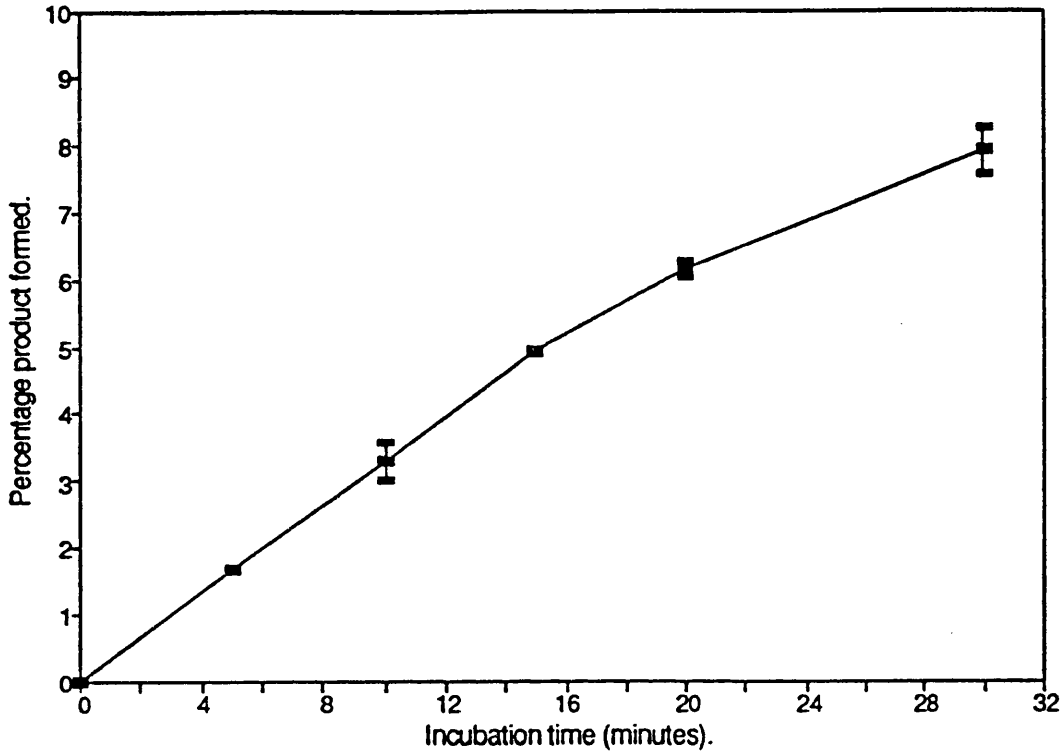


Figure 3.6. The percentage of hypoxanthine converted to product (IMP) as a function of assay time.

The reaction was carried out at 37°C for different incubation times. The 55°C heat treated partially purified PRPP synthetase preparation was used which had specific activity of 2.874 mUnits/mg protein and 0.32μg protein used in the standard assay.

100% substrate was 64358.2 cpm [^{14}C]Hypoxanthine in 70μl of the standard assay.

The error bars represent the standard error of the mean. Where the error bars are not obvious, they are encompassed within the markers. The markers represent the mean of two separate experiments carried out in dublicate.

there was always a standard assay carried out it was possible to account for loss of PRPP synthetase activity during the time required to complete all the runs and to standardise experiments carried out on different enzyme preparation batches.

3.4.14. The effect of inorganic pyrophosphatase (PPiase) to the overall reaction rate.

The amount of PRPP produced, equals the amount of PPi, since for every molecule of PRPP produced, one molecule each of IMP and PPi is formed as long as PRPP synthetase is the limiting enzyme reaction. From this result it is possible to speculate that the amount of PPi produced, which in this experiment amounted up to 0.2μM in the assay, was not high enough to cause any inhibition by reversing the HGPRTase reaction (Table 3.11).

Table 3.11. The effect of inorganic pyrophosphatase (PPiase) to the overall reaction rate.

Assay	- R5P	+ R5P	Δ (cpm)	[Protein] (μg/assay)
- PPiase	421.1	2188.85	1767.75	9.0
+ PPiase	348.6	2166.10	1817.50	9.0
- PPiase	197.9	1592.50	1394.60	4.5
+ PPiase	187.6	1434.70	1247.10	4.5

The total counts of hypoxanthine included in the standard assay were 56288 cpm, corresponding to a hypoxanthine concentration of 6.59 μM.

The standard assay was carried out with and without 10μl (2units) of PPiase solution. The enzyme solution consisted of a crude 20K sn enzyme preparation. The amount of PRPP produced, equals the amount of PPi, since for every molecule of PRPP produced, one molecule each of IMP and PPi is formed as long as PRPP synthetase is the limiting enzyme reaction. From this result it is possible to speculate that the amount of PPi produced, which in this experiment amounted up to 0.2μM in the assay, was not high enough to cause any inhibition by reversing the HGPRTase reaction.

3.4.15. Stability of PRPP synthetase in the presence and absence of inorganic phosphate (Pi) at 0°C.

The percentage of PRPP synthetase remaining after incubating at certain time intervals at 0°C is shown in Figure 3.7. The activity at the start of the incubation is denoted as 100%. The rate of loss of activity is very similar for both systems. At the start, the system in the absence of phosphate had only 60% of the activity as in its presence. So Pi activated the enzyme without stabilising it to any extent in the system used.

3.4.16. Stability of PRPP synthetase in the presence and absence of inorganic phosphate (Pi) at 37°C.

The results are shown in Figure 3.8. At 37°C PRPP synthetase quickly became inactive irrespective of the presence or absence of Pi in the storage buffer. Up to 80% of the activity of the enzyme was lost after the first hour of incubation at 37°C in the phosphate and TEA-HCl buffers used.

3.4.17. Stability of PRPP synthetase in the presence and absence of glycerol.

There was a rapid loss of activity in the first two days of storage with and without glycerol in the enzyme preparation (Figure 3.9). The rapid loss of activity continued in the absence of glycerol. Glycerol, at 20% (v/v) stabilises the enzyme for up to a week from the day of preparation and therefore was included in the end of all subsequent purification procedures which involved storage of the partially purified enzyme preparation prior to characterisation.

3.4.18. Stability of PRPP synthetase in the presence and absence of ATP in the protection buffer.

The activity remaining after a day's storage at 4°C is shown in Table 3.12.

On the day of preparation the addition of ATP in the standard assay appears to have an inhibitory effect on the activity of the enzyme. However, after a day's storage, it appears that loss of activity from the preparation which lacked

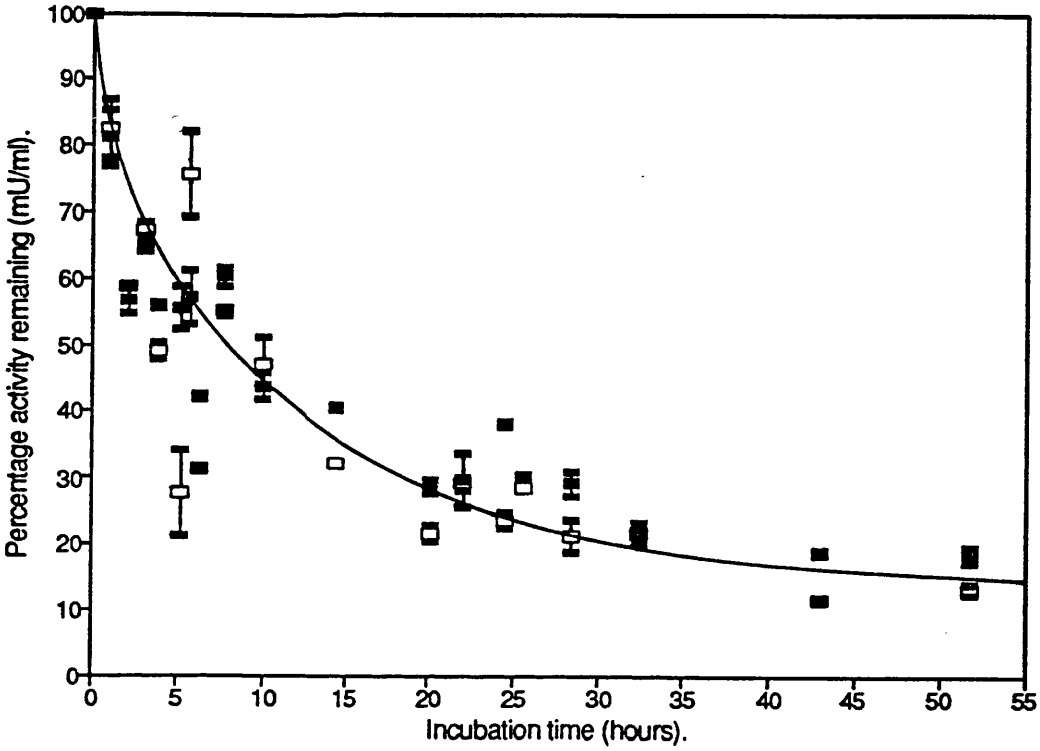


Figure 3.7. Stability of PRPP synthetase in the presence and absence of inorganic phosphate (Pi) at 0°C.

■——■ represent incubations in the presence of inorganic phosphate [20mM potassium phosphate (pH 7.6), 6mM $MgCl_2$, 0.1mM EDTA (disodium), 2.5mM mercaptoethanol and 0.5mM PMSF]. While, □——□ represent incubations with 20mM TEA-HCl (pH 7.6) instead of the phosphate.

Equal samples of 20K sn were passed through Pharmacia Sephadex G-25 columns pre-equilibrated in the appropriate buffer. The enzyme preparations were stored on ice. Aliquots of 10µl were assayed for enzyme activity using the standard assay. The error bars represent the standard error of the mean from duplicate results from two separate experiments. Where the error bars are not visible it is because they have been encompassed within the marker.

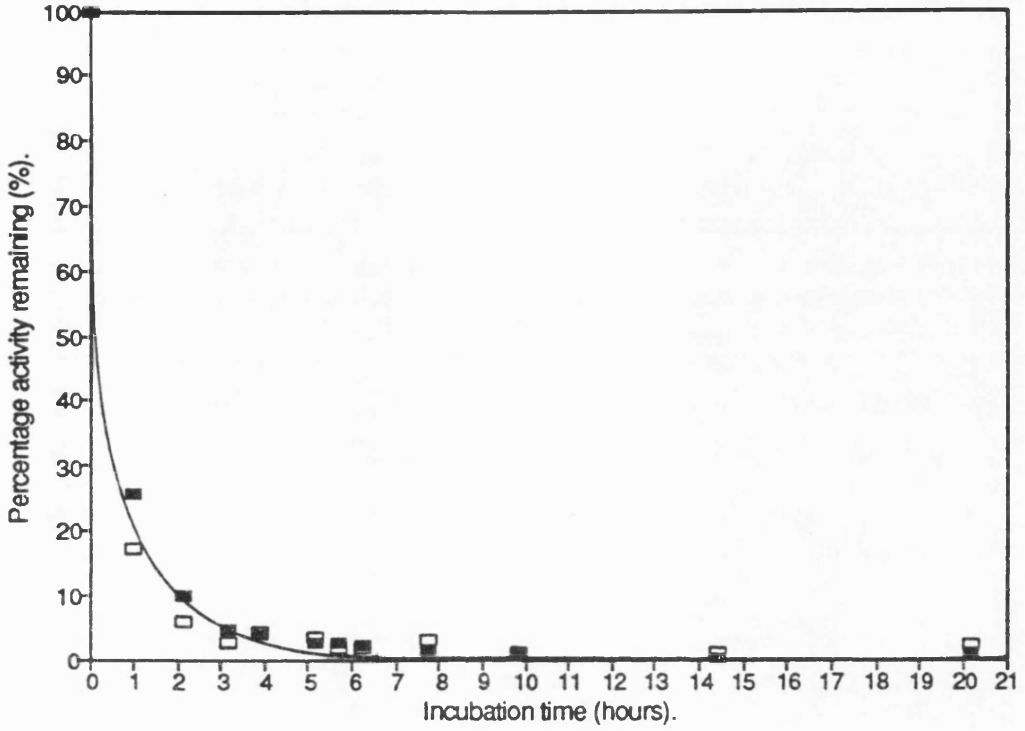


Figure 3.8. Stability of PRPP synthetase in the presence and absence of inorganic phosphate (Pi) at 37°C.

The enzyme preparations were incubated at 37°C in a temperature-controlled water-bath. The enzyme was quickly inactivated at 37°C irrespective of the presence or absence of inorganic phosphate in the incubation medium. The activity at the start of the incubation in the absence of phosphate was only 41% as in its presence. For more details see legend of Figure 3.7.

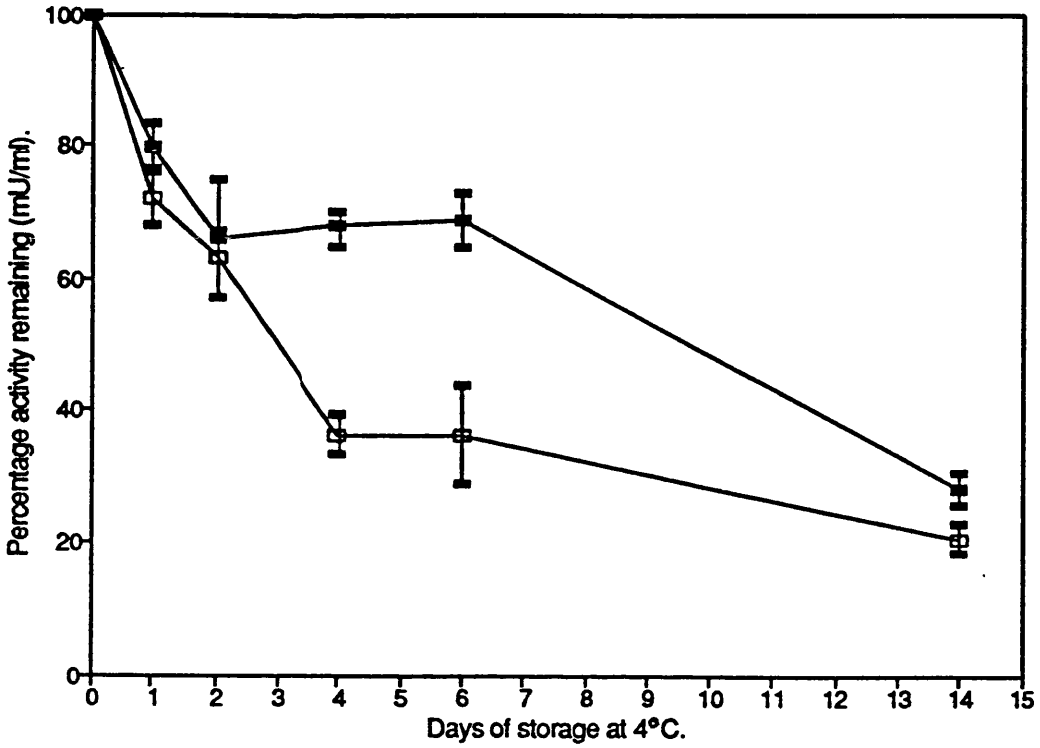


Figure 3.9. Stability of PRPP synthetase in the presence and absence of glycerol.

■——■ represent incubations in the presence of 20% (v/v) glycerol

□——□ represent the absence of glycerol from the protection buffer.

The bars represent the standard error of the mean. When the error bars are not visible, they are encompassed within the marker.

Concentrated heat treated (5 minutes at 55°C) enzyme preparation (specific activity of 3.676 mUnits/mg protein and 1.36 mUnits/ml) had been dialysed against protection buffer with and without 20% (v/v) glycerol and stored at 4°C. The samples were diluted 10x in protection buffer and 10µl assayed using the standard assay. Error bars stand for standard error of the mean.

Table 3.12. Stability of PRPP synthetase in the presence and absence of ATP in the protection buffer.

The 5 minute 55°C treated sn preparation was stored in the presence and absence of 0.3mM ATP in protection buffer and kept at 4°C overnight.

The activity remaining after a day's storage at 4°C is shown in the table below:

Day	System	Specific activity (mUnits/mg protein)	Remaining activity (%)
0	- ATP	5.728	100.00
0	+ ATP	4.201	100.00
1	- ATP	1.970	34.39
1	+ ATP	1.973	46.97

ATP in the buffer was greater than that with ATP. It would seem therefore that ATP has a stabilising effect on the enzyme although the extent seems not to be so pronounced since both enzyme preparations lost up to 50% of the enzyme activity during overnight storage at 4° C in phosphate protection buffer, pH 7.6.

3.4.19. The effect of the presence or absence of EDTA in the presence and absence of Pi.

EDTA (disodium) was excluded from both the protection and the standard assay cocktail, in order to see the effect its absence might have on the reaction rate observed.

From the results in Table 3.13, it appears that the presence of phosphate in the enzyme buffer causes the enzyme activity to remain equally high in the presence and absence of EDTA and phosphate in the assay. The absence of phosphate in the enzyme preparation, causes the loss of about 50% of the activity but additional absence of EDTA causes an extra 10% loss of enzyme activity. Phosphate and EDTA have synergistic effects in activating the enzyme.

3.4.20. The effect of inorganic phosphate (Pi) on activity of trypanosomal PRPP synthetase.

In order to assess the effect of inorganic phosphate on enzyme activity (as opposed to stability) the enzyme was assayed at various concentrations of Pi. The results are given in Figure 3.10.

It was repeatedly observed that the PRPP synthetase activity decreased rapidly at phosphate concentrations higher than 40mM. In order to see if this effect was due to enzyme inhibition by inorganic phosphate, the experiment was repeated with rat liver enzyme. PRPP synthetase requires phosphate for activity and for the rat liver enzyme the activity should increase with increasing phosphate concentration up to a maximum at 100mM (Roth *et al.*, 1974). It was repeatedly observed that the PRPP synthetase activity decreased rapidly at phosphate concentrations higher than 40mM, irrespective of the source of PRPP

Table 3.13. The effect of the presence or absence of EDTA in the presence and absence of Pi.

Half the enzyme preparation had been passed through a Sephadex G-25 column pre-equilibrated with TEA-HCl protection buffer in order to investigate the effect of EDTA in the absence of Pi. The results obtained were tabulated below:

System	+ Pi + EDTA	- Pi - EDTA	- Pi + EDTA
Enzyme buffer with Pi	100.00	102.6	107.24
Enzyme buffer with TEA-HCl	47.78	34.6	48.08

The enzyme preparation used in this experiment was a partially purified preparation with specific activity of 6.93 mUnits/mg protein.

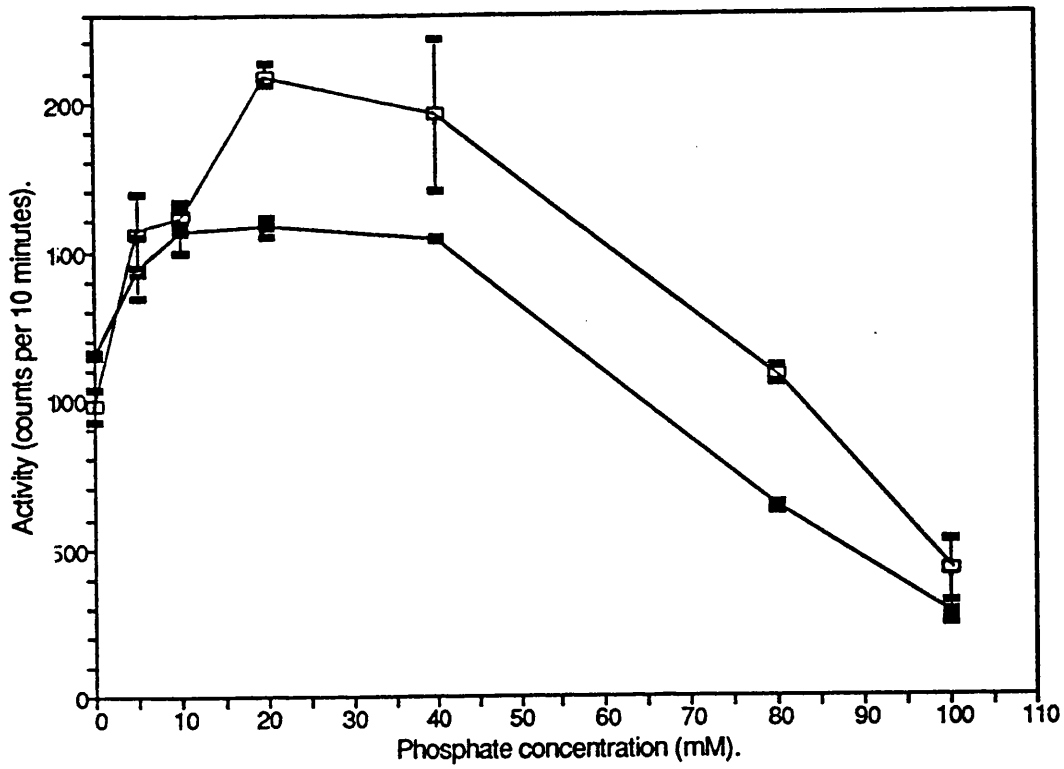


Figure 3.10. The effect of inorganic phosphate (Pi) on activity of trypanosomal PRPP synthetase. Potassium phosphate solutions were made up in 50mM TEA-HClbuffer, pH 7.6 and the pH adjusted when neccesary to 7.6. PRPP synthetase activity was corrected for the blank (no R5P in the assay). ■—■ represent the trypanosomal PRPP synthetase system (specific activity 0.288 mUnits/mg protein; 5.87 μM hypoxanthine concentration in the assay). While, □—□ represent the rat liver PRPP synthetase system (specific activity 1.465 mUnits/mg protein; 4.32 μM hypo-xanthine concentration in the assay). The trypanosomal PRPP synthetase preparation (4.5 μg protein /assay) consisted of a crude homogenate whose buffer was exchanged for 50mM TEA-HCl (pH 7.6) through passage through a Sepharose G-25 column. The rat liver PRPP synthetase preparation consisted of a crude 100K*xg* supernatant (1.17 μg protein/assay), whose buffer was also exchanged for 50mM TEA-HCl (pH 7.6) through passage through a Sepharose G-25 column. The standard assay was carried out with 10μl of diluted enzyme preparations. The error bars represent the standard error of the mean of three separate experiments. Where the error bars are not visible, they are encompassed within the markers.

synthetase. From this result it was possible to exclude the possibility of PRPP synthetase inhibition as a potential cause for the decrease in activity observed and to assign it to an inherent limitation of the standard assay, likely to be due to competition of the Pi with IMP binding onto the DE-81 filters.

It was realised that the Pi effect may have been observed by an experimental artefact resulting from interference of Pi in binding of IMP to the DEAE filters in the assay. This was investigated by testing for the effect of Pi in the binding of IMP (Table 3.14) and the effect of altering the protocol of filters for the effect of the number of filters on the binding of IMP in the presence of Pi (Table 3.15). The effect of pre-soaking the filters in 4mM hypoxanthine solution in 20mM phosphate buffer (pH 7.6) was also investigated for its effect on the binding of IMP to the filters (Table 3.16).

From the data in Table 3.14 it is clear that Pi interferes with [^{14}C] IMP binding on the DE-81 filters.

With just IMP added to the filters, there is an improvement in binding with the stacked filters which is reduced by addition of Pi. It might be possible to improve the binding of the IMP onto the filters if the filters were not pre-soaked in the adenosine in 20mM potassium phosphate (pH 7.6) buffer before use (See Table 3.16).

It was found (Table 3.16) that the non-specific binding of [^{14}C]hypoxanthine was high with filters which were not pre-soaked, but not more than 0.5% of the total counts filtered through as opposed to the 0.3% of the total counts in non-specifically bound onto the pre-soaked filters. The stacking of 3 unsoaked filters was therefore used in Figure 3.10, as the binding improved to 95% of the total IMP counts produced under those conditions.

It was suggested that a callibration curve of % CPM bound at different concentrations of Pi be constructed, so that the effect of the interference of Pi with IMP binding might be corrected for.

Table 3.14. The effect of phosphate on IMP binding onto the DE-81 disc.

A standard assay was set up as usual and allowed to proceed with the formation of [^{14}C] IMP. The reaction was stopped by heating in a boiling water-bath for 3 minutes and the denatured proteins spun down and removed.

A suitable sample was deposited into Eppendorf tubes and to a third of the tubes, 100mM phosphate was added (assay+Pi) and to the third 100mM phosphate and 10µl of 0.1 M cold IMP (assay+IMP+Pi) added and to the third 10µl of 0.1 M cold IMP was added (assay+IMP) as usual. The contents of the tubes were filtered through DE-81 filters and the results obtained as follows:

System	cpm on filter	% total cpm
assay+IMP	1075.1	100.00
assay+IMP+Pi	689.4	64.12
assay+Pi	743.3	69.14

Table 3.15. The effect of the number of filters on the amount of [^{14}C] IMP binding in the presence of Pi.

A suitable amount of [^{14}C] IMP was produced as described in Table 3.14. The same number of counts were passed through one and through 3 filters both stacked and separate (one third of the sample each) and the results were as follows:

System	cpm on filter	% total cpm
one filter	4512.7	63.51
3 stacked	7105.6	100.00
1/3 on 3 filters in total	5979.6	84.15
with added Pi and cold IMP,		
one filter	2793.6	39.30
3 stacked	5706.4	80.31
1/3 on 3 filters in total	4342.3	61.10

Table 3.16. The effect of pre-soaking of filters to the binding of IMP.

The filters were not soaked in the adenosine solution in 20mM phosphate buffer, pH 7.6 as usual, but used directly from the packet to see if that would make any difference to the IMP binding onto the DE-81 filters.

System	cpm on filter which was soaked	cpm on filter which was not soaked	total cpm (%)
one filter,			
no Pi	1876.2	2137.6	100.00
+ Pi	1409.2	1594.2	75.00
3 stacked filters,			
no Pi	2421.9	2589.9	100.00
+ Pi	2238.4	2537.2	95.00

3.4.21. Substrate specificity of *T.b.brucei* PRPP synthetase.

Nucleotide specificity.

From the results in Table 3.17 *T.b.brucei* PRPP synthetase is highly specific for ATP as the pyrophosphoryl donor. Except for dATP which can replace ATP (relative activities 100:75), the other nucleotides (NTP) tested, *viz.*, GTP, CTP, and UTP, gave less than 1% of the activity as compared to ATP. With ITP the rate was within approximately 80% of the rate with ATP, however, the blank (no R5P) was as high as the test, which was equal to about 71% of the activity as compared to ATP. The difference resulted in percentage activity for ITP of 7.7% compared to that with ATP as substrate.

Pentose phosphate specificity.

The investigation of pentose phosphate specificity of trypanosomal PRPP synthetase was impared due to the lack of pure enzyme, and the limitations of the coupling assay. However, the conversion of ribulose-5-phosphate and ribose-1-phosphate directly into PRPP or first into ribose-5-phosphate was investigated.

Ribulose-5-phosphate as substrate for trypanosomal PRPP synthetase.

From Table 3.18, it appeared that the assay system converted ribulose-5-phosphate to IMP with the same rate as it converted R5P. The extent of product formation increased with the time of incubation in a linear way. From these results it appeared that the trypanosomal PRPP synthetase preparation may have had contaminating D-ribose-5-phosphate ketol-isomerase activity or that ribulose-5-phosphate acted as a substrate with similar affinity to R5P.

Ribose-1-phosphate as substrate for trypanosomal PRPP synthetase.

In this experiment ribose-5-phosphate (standard assay concentration 2.87mM) was substituted with ribose-1-phosphate (1mM in the assay) in the standard assay. A crude trypanosomal extract was used. There was a small increase in the activity of the blank (no R5P in the assay) when ribose-1-

Table 3.17. Nucleotide triphosphate specificity of *T.b.brucei* PRPP synthetase.

The standard assay was carried out with dATP, CTP, GTP, ITP and UTP at final concentration of 0.302 mM [$MgNTP^{2-}$] in the assay. Excess $MgCl_2$ concentration of 10 mM in the assay was used.

The results were expressed as % of PRPP formed as compared to that observed with ATP which was 8.46 nmoles PRPP formed/5 minutes. Each incubation medium contained 0.6 μg protein.

NTP	+ R5P	- R5P	Δ (cpm)	% ATP
dATP	2182.4 1928.0	118.8 113.0	1939.3	75.000
UTP	98.8 81.6	65.0 85.6	14.9	0.576
CTP	65.8 52.2	63.0 51.8	1.6	0.062
ITP	2123.0 1968.8	1869.0 1824.6	199.1	7.700
GTP	67.2 82.4	57.4 53.4	19.4	0.750

Table 3.18. Ribulose-5-phosphate as substrate for trypanosomal PRPP synthetase.

The data were obtained with Ribulose-5-phosphate, at 1mM concentration in the assay. For Ribose-5-phosphate, the standard assay concentration of 2.87mM was used. In the blank assays the sugar phosphate is omitted from the incubation mixture.

Time of assay	Test	Blank (cpm)	Δ (cpm) (cpm)	Activity (μUnits)
with Ribulose-5-phosphate.				
10 minutes	4906.5	231.9	4677.4	3.831
	5042.0	226.3	4812.9	3.942
30 minutes	15535.5	349.3	15202.7	12.451
	12673.5	316.3	12340.7	10.107
with Ribose-5-phosphate.				
10 minutes	4329.9	224.6	4119.95	3.374
	4723.1	195.3	4513.15	3.696

The standard assay contained [^{14}C]hypoxanthine concentration of 9μM and 2.16μg of partially purified enzyme preparation with specific activity of 1.527 nmoles PRPP formed/minute/mg protein, in the standard assay.

phosphate was present (from 307 to 864 counts on the DE-81 filters per 10 minutes), but the results were not clear. A small increase in the blank was not conclusive of ribose-1-phosphate being the substrate for PRPP synthetase and the activity observed could have been due to some ribose-5-phosphate contamination of the ribose-1-phosphate preparation.

3.4.22. The effect of Mg^{2+} concentration on HGPRTase activity.

From Figure 3.11 it is seen that HGPRTase requires Mg^{2+} for activity as reported already for phosphoribosyltransferases. The activity increased with increasing Mg^{2+} concentration with plateau at Mg^{2+} concentration higher than 5mM in the assay.In the standard assay for PRPP synthetase activity Mg^{2+} is included at a final concentration of 5mM.

3.4.23. The effect of cations on HGPRTase activity.

From the data in Table 3.19 it could be seen that HGPRTase activity with magnesium was as high as with calcium. Manganese however seemed to activate the enzyme 3 fold while cobalt (divalent) also activated HGPRTase by increasing its activity 2 fold to that with magnesium. Copper, nickel and zinc were less effective at substituting for magnesium and inactivated the enzyme by 20%. The effect of the different cations on HGPRTase was therefore not prohibiting the investigation of the effect of cations on trypanosomal PRPP synthetase.

3.4.24. The effect of cations on trypanosomal PRPP synthetase.

In order to make sure that the effect observed was due to the PRPP synthetase reaction, two concentrations of PRPP synthetase enzyme preparation were assayed and only when the doubling of the enzyme resulted in twice the amount of product being produced the result was considered valid. Unfortunately it was very difficult to reproduce these results and therefore care is needed in their interpretation.

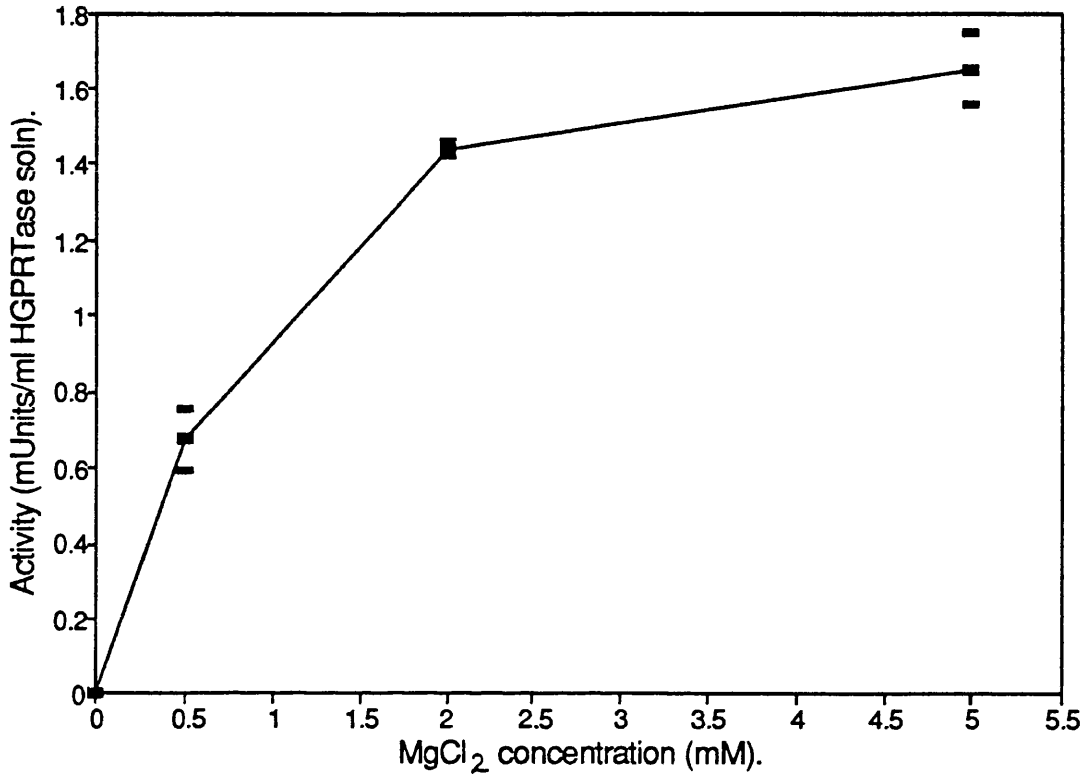


Figure 3.11. The effect of Mg^{2+} concentration on HGPRTase activity.

In a final volume of 70µl, the concentration of reagents was: 20mM potassium phosphate, pH 7.6, 0.143mM EDTA, 7.5µM [^{14}C]hypoxanthine, 10mM PRPP, 1munit of HGPRTase, Mg^{2+} at 0, 0.5, 2 and 5mM and water to make up the volume. The reaction was initiated by the addition of HGPRTase and carried out at 37°C for 10 minutes.

The results were obtained after subtraction of the activity in the control assay which was carried out without any PRPP. The presence of divalent cations such as Mg^{2+} was compulsory for activity. The rate of HGPRTase reaction increases with increasing Mg^{2+} concentration to a plateau at > 5mM Mg^{2+} in the assay.

Table 3.19. The effect of cations on the HGPRTase activity.

All metals are used as the chloride salt and are present in the HGPRTase assay at 2mM (see legend of Figure 3.11. for assay details). The $MgCl_2$ sample gave 4.21 pmoles PRPP per 10 minutes. Values shown for other divalent cations represent percent activity in the assay in which divalent cation has supplimented $MgCl_2$, compared to the control assay with 2mM $MgCl_2$ alone.

Metal ion.	Activity.
Magnesium	100.0
Manganese	314.4
Calcium	104.4
Cobalt(ous)	196.6
Copper	78.1
Nickel	84.6
Zinc	87.2

The effect of cations on trypanosomal PRPP synthetase is presented in Table 3.20. For the trypanosomal PRPP synthetase, the activity with magnesium was maximal and similar to that with manganese as for PRPP synthetases from other sources studied to date. Nickel had a very high inhibitory effect. The inhibitory effect of zinc and cobalt was nearly 50%.

3.4.25. Kinetic studies of trypanosomal PRPP synthetase.

The partially purified enzyme preparation was used for the investigation of the kinetic behaviour of trypanosomal PRPP synthetase. The activity and stability of the enzyme was sufficient for kinetic studies to be carried out. Each point was repeated in duplicate, with less than 5% difference between the two repeats.

The validity of the kinetic studies was checked by assaying two concentrations of enzyme preparation for every parameter changed and only when the doubling of the enzyme concentration resulted in the doubling of counts (product) formed, were the results accepted as valid.

3.4.26. Considerations for experimental protocol with $MgATP^{2-}$ as substrate.

It is well-known that most enzymes that are ATP-dependent also require Mg^{2+} for formation of the true substrate, $MgATP^{2-}$. It is impossible to prepare a solution of pure $MgATP^{2-}$ without a number of other ions also being present. An equimolar mixture of ATP and $MgCl_2$ at pH 7 contains appreciable proportions of $MgATP^{2-}$, ATP^{4-}, $HATP^{3-}$, Mg^{2+} and Cl^-, as well as traces of $MgHATP^-$, Mg_2ATP and $MgCl^+$. Moreover, the proportions of each species vary with the total $MgCl_2$ and ATP concentrations, the pH, the ionic strength and the concentrations of other species (such as buffer components) that may be present (Cornish-Bowden, 1979). When studying the effect of increasing $MgATP^{2-}$ concentrations in the assay, it is important to ensure that the variation in the Mg^{2+} and ATP^{4-} concentrations is minimal.

Table 3.20. The effect of cations on trypanosomal PRPP synthetase.

All metals are used as the chloride salt and present in the standard assay at 2mM final concentration. The $MgCl_2$ sample gave 46.3pmoles PRPP per 10 minutes. The specific activity of the partially purified preparation after heat treatment, dialysis and concentration, measured with standard assay (5mM Mg^{2+}) was 3.32mUnits/mg protein. Values shown for other divalent cations represent percent activity in the assay in which divalent cation has supplimented $MgCl_2$, compared to the control assay with $MgCl_2$ alone.

Metal ion.	Activity.
Magnesium	100
Manganese	87-98
Calcium	40-76
Cobalt(ous)	23-50
Copper	160-200
Nickel	2
Zinc	17-30

A 'badly' designed experiment involves the variation of the total concentrations of ATP and $MgCl_2$ in constant ratio. Whether this ratio is 1:1 or any other, it leads to wild variations in the proportion of ATP existing in any particular form, and should be avoided. Somewhat less objectionable, is to keep the total $MgCl_2$ concentration constant at a value that exceeds the highest ATP concentration by about 2-5 mM. Although this design does ensure that ATP exists largely as $MgATP^{2-}$, it can produce undesirably large variations in the concentrations of free Mg^{2+} and of Mg_2ATP.

Storer and Cornish-Bowden (1976) found that ATP exists in a high and nearly constant proportion (about 80%) as $MgATP^{2-}$ in solutions in which the total $MgCl_2$ concentration exceeds the total ATP concentration by 1-10 mM. This was taken into consideration in the kinetic studies of trypanosomal PRPP synthetase and the concentration of Mg^{2+} exceeded the concentration of ATP by 1 or 2mM throughout these experiments.

3.4.27. Substrate saturation experiments with trypanosomal PRPP synthetase with $MgATP^{2-}$ as the varying substrate.

In this investigation the total $MgATP^{2-}$ concentration in the assay was varied while keeping the R5P concentration fixed at 2.87mM and the Mg^{2+} concentration in excess of the ATP concentration in the assay. The $MgATP^{2-}$ concentration was calculated as 84.1% of the ATP added, since Mg^{2+} was in excess by 2mM over the ATP concentration in the assay.

The rate of reaction of trypanosomal PRPP synthetase increased with increasing $MgATP^{2-}$ concentrations up to about 0.35 mM $MgATP^{2-}$, after which the reaction rate decreased. The data were fitted to a standard substrate inhibition equation, $v = Vmax/(1 + Km/[S] + [S]/Ki)$, and presented in Figure 3.12. From a computer program for high substrate inhibition written by Ian Goodyear, Biochemistry Department, Bath University, the kinetic parameters for trypanosomal PRPP synthetase were estimated to be Km^{app} $MgATP^{2-}$ of 63μM,

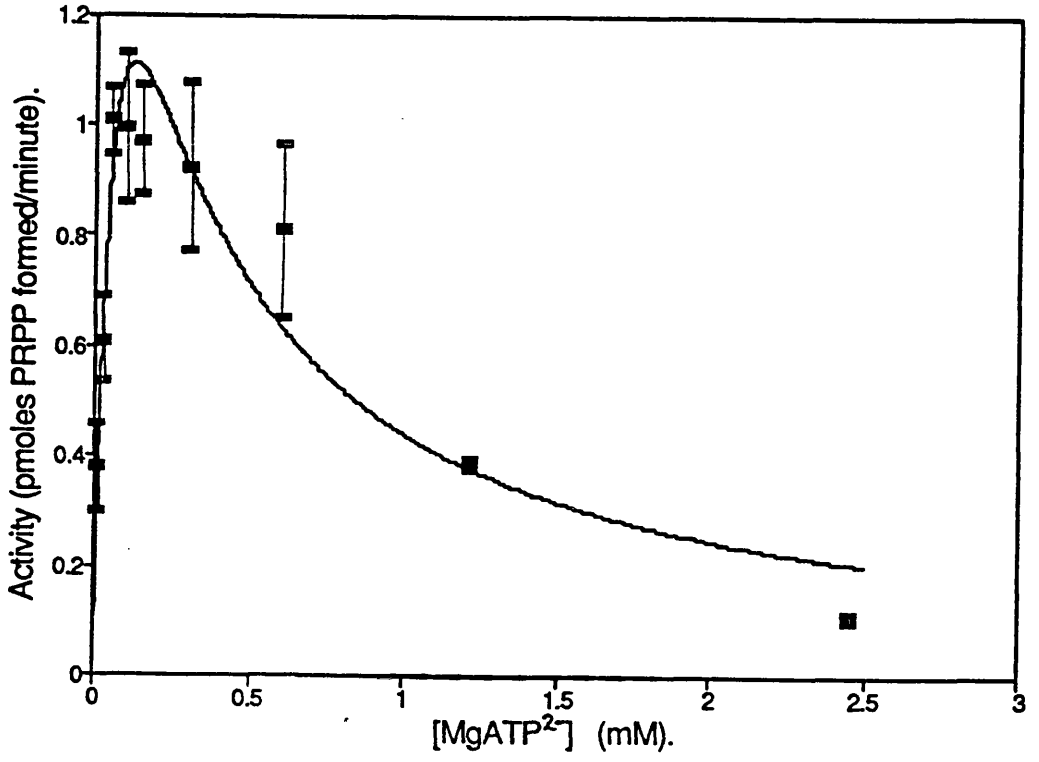


Figure 3.12. Substrate saturation experiments with trypanosomal PRPP synthetase with $MgATP^{2-}$ as the varying substrate.

The investigation was carried out using partially purified enzyme preparation (1.23μg protein in the assay with specific activity of 1.024 mUnits/mg protein in the standard assay) , and 8μM [^{14}C]hypoxanthine in the assay. The experiment was done with fixed R5P concentration of 2.87mM and 2mM $MgCl_2$ (excess of ATP) concentration, using the standard assay, with TEA-HCl 20mM (pH 7.6) instead of the usual 20mM KH_2PO_4 (pH 7.6) buffer.

Ki^{app} $MgATP^{2-}$ of 0.25mM and $Vmax^{app}$ of 2.23 munits enzyme/ml enzyme preparation (specific activity of PRPP synthetase of 2.04 munits/mg protein).

3.4.28. Substrate saturation experiments with trypanosomal PRPP synthetase with R5P as the varying substrate.

Figure 3.13 shows the substrate saturation curve for ribose-5-phosphate. The data were fitted to the Michaelis-Menten enzyme rate equation, v= Vmax [S]/([S]+Km) and the apparent kinetic parameters were estimated using the Direct-Linear plot of Eisenthal and Cornish-Bowden (1974). The calculated Km^{app} of trypanosomal PRPP synthetase for R5P was 27 ± 9μM for the trypanosomal PRPP synthetase. The calculated Vmax of trypanosomal PRPP synthetase for R5P was 1.55 ± 0.3 munits/mg protein in the partially purified PRPP synthetase preparation.

It was considered as unlikely that the physiological concentration of R5P would be higher than 5 mM so this was the highest R5P concentration assayed.

3.4.29. Effect of Mg^{2+} on enzyme activity.

The efect of Mg^{2+} concentration on the activity of the partially purified trypanosomal PRPP snthetase was investigated at a R5P concentration of 2.87mM and 3 fixed $MgATP^{2-}$ concentrations. At this R5P concentration the activity is maximal as shown in Figure 3.13. A magnesium concentration of 0mM excess denotes that magnesium was present in the assay at the same concentration as ATP (1.43mM, the concentration of ATP of the standard assay). The concentrations of $MgATP^{2-}$ were chosen to represent different aspects of the substrate/activity profile as shown in Figure 3.12. From figure 3.12 it is seen that $MgATP^{2-}$ concentration at 0.0202 mM and 0.101 mM is at the ascending branch of the high substrate inhibition curve while 2.06mM $MgATP^{2-}$ is at an inhibitory concentration of $MgATP^{2-}$.

It was found that excess Mg^{2+} (above ATP concentration) was inhibitory. The activity of PRPP synthetase was plotted against excess

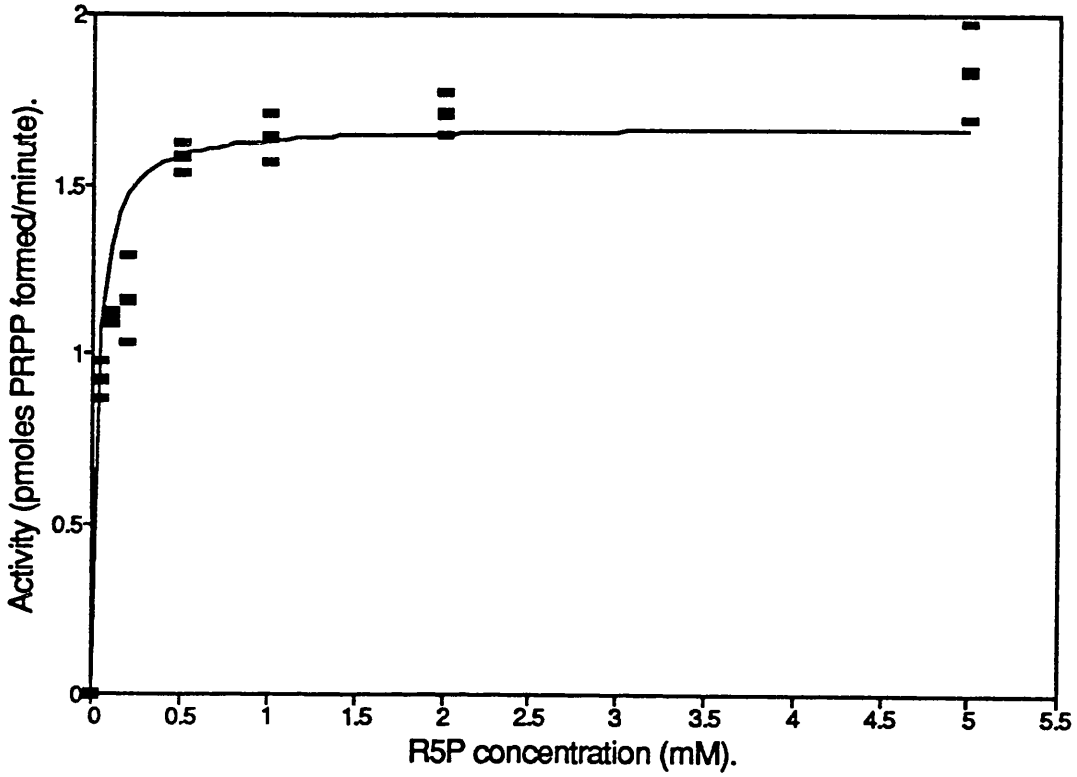


Figure 3.13. Substrate saturation experiments with trypanosomal PRPP synthetase with R5P as the varying substrate.

The magnesium concentration throughout the assay was kept at 1mM excess over the [$MgATP^{2-}$] concentration which was constant at 0.101 mM at all the R5P concentrations assayed. The absence of [$MgATP^{2-}$] constituted the blank. The assay was carried out for 10 minutes at 37°C as usual. The specific activity of the partially purified PRPP synthetase used was 1.93 munits/min/mg protein and 1.08µg protein was included in each assay.

The data were fitted to the Michaelis-Menten enzyme rate equation, v= Vmax [S]/([S]+Km) and the kinetic parameters were estimated using the Direct-Linear plot of Eisenthal and Cornish-Bowden (1974).

magnesium concentration and presented in Figure 3.14. The results were expressed as fractional inhibition, i, defined as:

$$i = 1 - \frac{v_0^i}{v_0}$$

where v_0^i and v_0 are the reaction rates in the presence and absence of inhibitor respectively.

The insert to Figure 3.14 shows the results plotted as fractional inhibition against excess magnesium concentration at the 3 fixed $MgATP^{2-}$ concentrations. The calculated values for the 'operational' inhibition constant are presented in Table 3.21. It appears that the decrease in activity of the partially purified PRPP synthetase preparation did not differ (within experimental error) with the concentration of $MgATP^{2-}$ in the assay.

The results were plotted as activity *versus* $MgATP^{2-}$ concentration at the five excess magnesium concentrations and presented in Figure 3.15 as hyperbolic curves after the data had been fitted to the Michaelis-Menten enzyme rate equation as for the substrate saturation graphs. The computer programme was also used to obtain the apparent kinetic parameters, Km and Vmax from the Direct Linear Plot of Eisenthal and Cornish-Bowden (1974). The apparent kinetic parameters Km and Vmax so obtained are presented in Table 3.22. From the data in Table 3.22 it appears that excess magnesium (over ATP) is a competitive inhibitor with respect to $MgATP^{2-}$, since increasing excesses of magnesium gave unaltered (within experimental error) apparent Vmax values and increasing apparent Km values. The secondary plots of the ratio of apparent Km and Vmax against excess magnesium concentration and of the reciprocal of the apparent Vmax against excess magnesium concentration are presented in Figures 3.16 and 3.17 respectively. From figure 3.16, a plot of apparent Km'/Vmax' against Mg^{2+} excess, a straight line was obtained with x-axis intercept of apparent -Ki of -1.2 ± 0.8 mM. From Figure 3.17 of the secondary

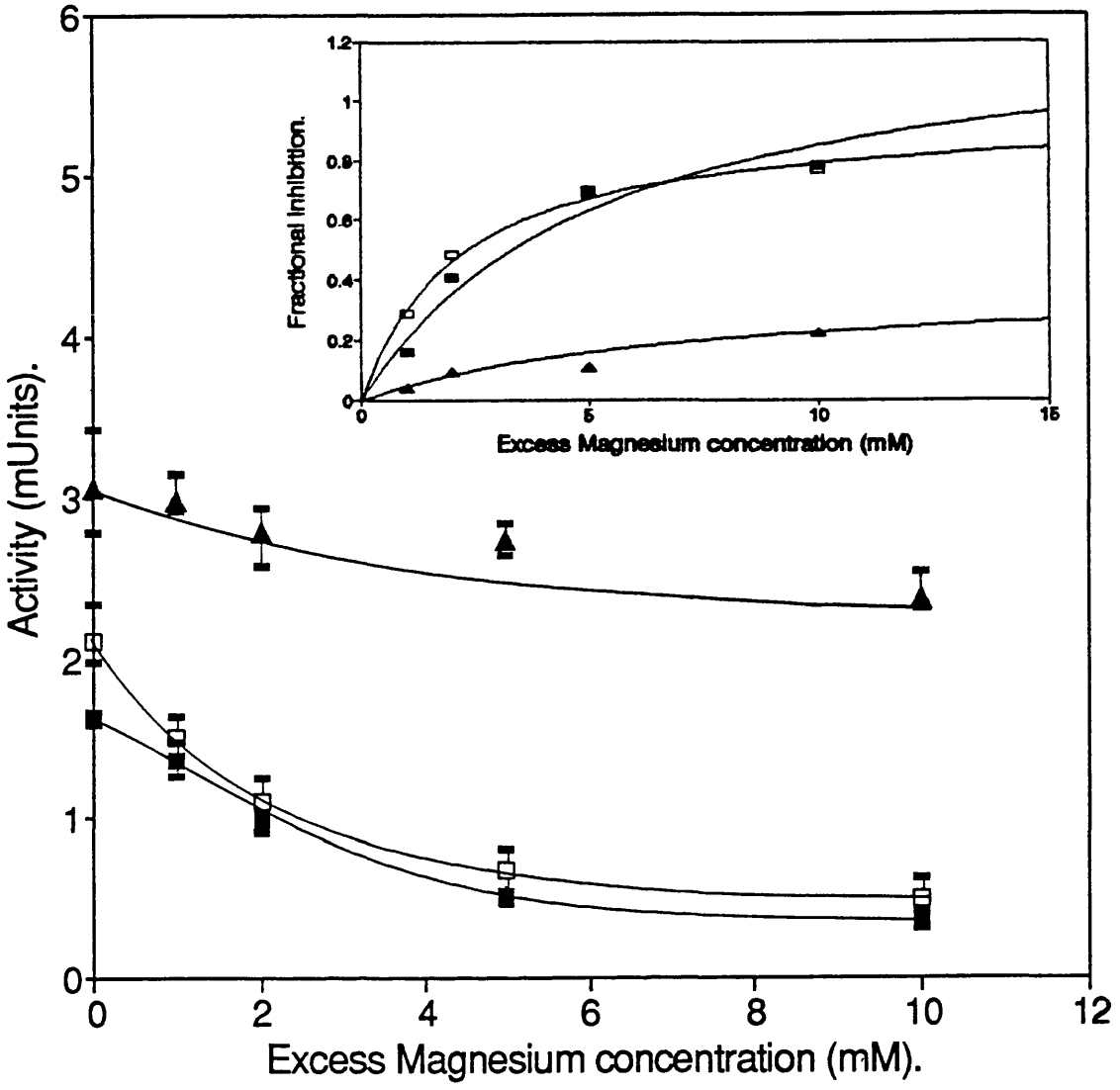


Figure 3.14. Activity of trypanosomal PRPP synthetase plotted against excess Mg^{2+} concentration in the assay at 3 fixed concentrations of $MgATP^{2-}$.

The [R5P] was fixed at 2.87mM throughout the experiment.

■——■ represent the assay with [$MgATP^{2-}$] = 0.0202mM,

□——□ represent the assay with [$MgATP^{2-}$] = 0.101mM and

▲——▲ represent the assay with [$MgATP^{2-}$] = 2.06mM.

In the insert the data are presented as fractional inhibition plotted against excess [Mg^{2+}]. The curves were fitted by computer to the Michaelis - Menten enzyme rate equation.

Table 3.21. The 'operational' inhibition constant of excess magnesium concentration for trypanosomal PRPP synthetase.

[R5P] (mM)	[$MgATP^{2-}$] (mM)	'Operational' inhibition constant (mM)
2.87	0.0202	5.320 ± 1.485
2.87	0.1010	2.150 ± 0.780
2.87	2.0600	7.410 ± 5.730

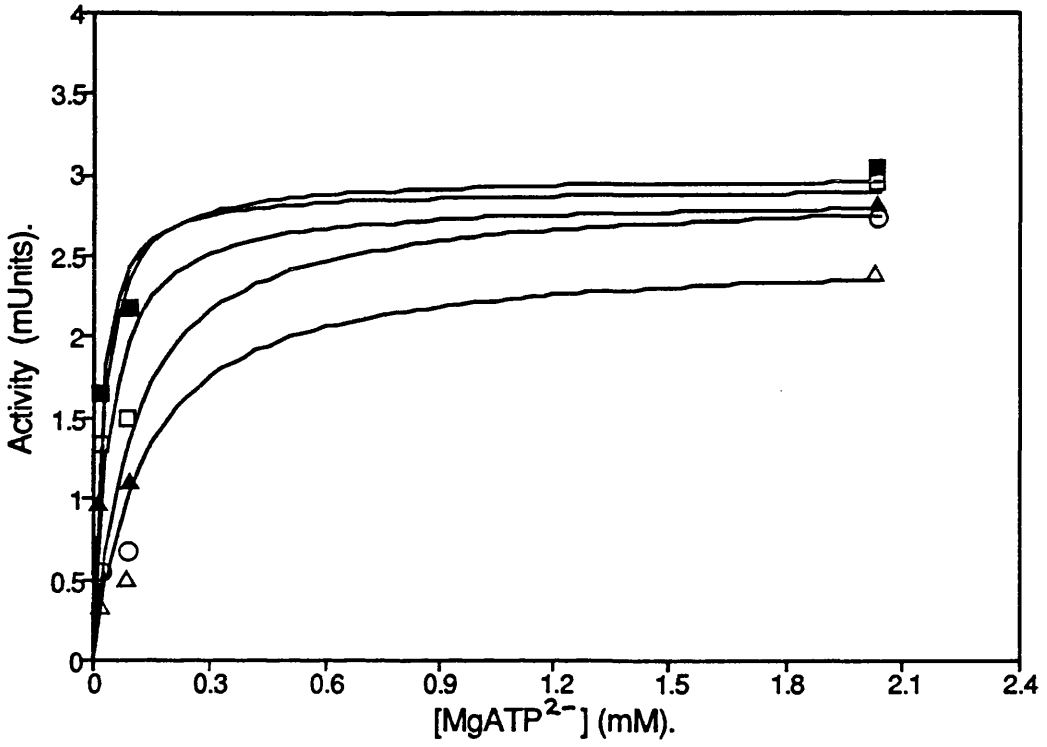


Figure 3.15. Activity of trypanosomal PRPP synthetase plotted against $MgATP^{2-}$ concentration. The curves were fitted by a computer program for Michaelis - Menten rate equation with apparent Km and Vmax values calculated from the Direct Linear Plot as quoted in text.

■——■ represent the assay with 0mM Mg^{2+} excess over [ATP],

□——□ represent the assay with 1mM Mg^{2+} excess over [ATP],

▲——▲ represent the assay with 2mM Mg^{2+} excess over [ATP],

○——○ represent the assay with 5mM Mg^{2+} excess over [ATP] and

△——△ represent the assay with 10mM Mg^{2+} excess over [ATP].

Table 3.22. The calculated apparent kinetic parameters for inhibition by five different excess magnesium concentrations over ATP concentration.

Excess $[Mg^{2+}]$ (mM)	Km^{app} (mM)	$Vmax^{app}$ (mUnits)
0	0.0179 ± 0.0045	2.91 ± 0.115
1	0.0266 ± 0.0053	2.99 ± 0.060
2	0.0390 ± 0.0077	2.84 ± 0.115
5	0.1010 ± 0.0940	2.88 ± 0.125
10	0.1280 ± 0.0329	2.48 ± 0.145

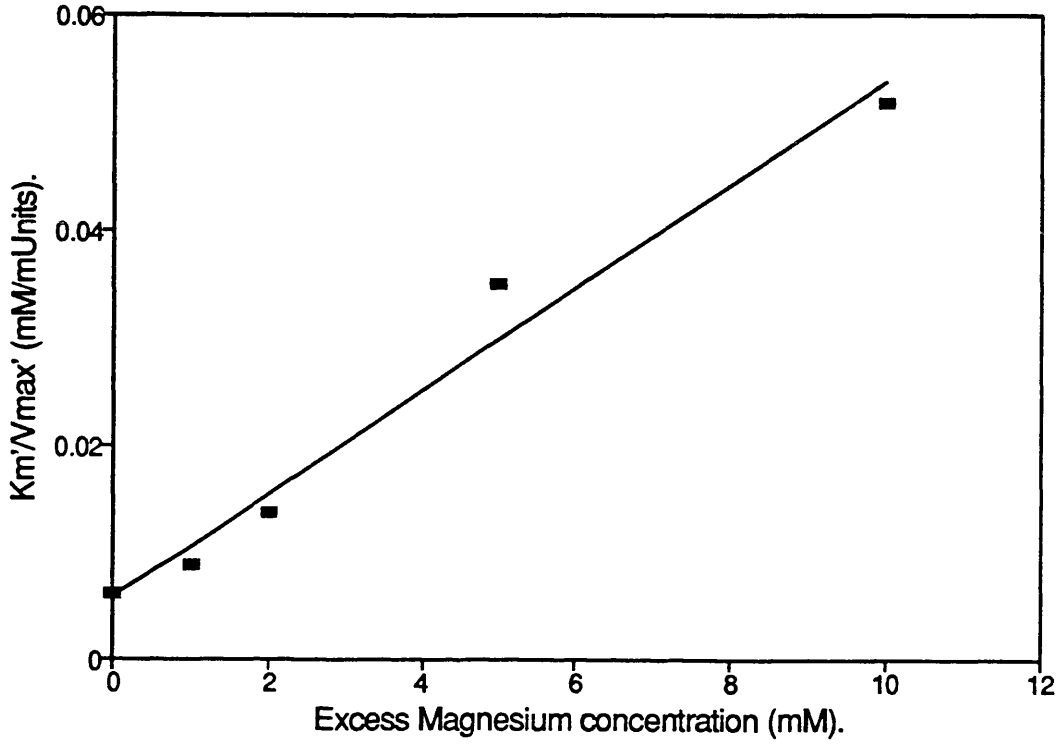


Figure 3.16. Secondary plot of apparent Km/Vmax (from Direct Linear plot estimates of data presented in Figure 3.15) against excess Mg^{2+} concentration over ATP concentration in the assay.

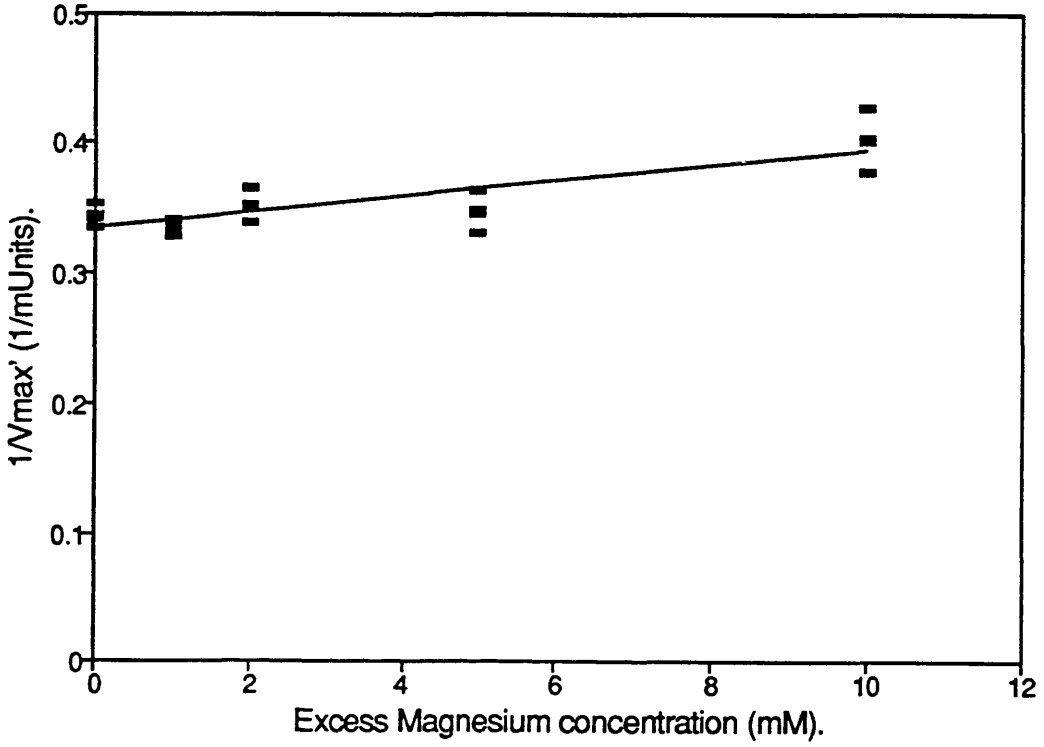


Figure 3.17. Secondary plot of reciprocal of apparent Vmax (from Direct Linear plot estimates of data presented in figure 3.15) against excess Mg^{2+} concentration over ATP concentration in the assay.

plot of $1/Vmax^{app}$ the estimate of the inhibition constant Ki' for the enzyme-substrate complex was obtained as 54.9 ± 15 mM.

The free concentration of magnesium was likely to have been overestimated since no consideration was given to the binding of magnesium to R5P and PRPP, as well as for the magnesium interaction with the coupling enzyme HGPRTase. The discrepancy in the concentration of free magnesium is likely to be small and fairly constant so patterns gave a reasonable approximation to the true effect of free Mg^{2+} on trypanosomal PRPP synthetase.

3.4.30. ADP inhibition studies of trypanosomal PRPP synthetase.

ADP inhibition was investigated at two fixed concentrations of each substrate while varying the other. The $MgATP^{2-}$ concentrations were 0.036mM and 0.36mM, chosen so as to represent ascending and maximal activities respectively as shown in Figure 3.12. The R5P concentrations chosen were 0.057mM (near the Km) and 2.87mM (saturating) (See Figure 3.13). The assays were essentially the standard assay but with 10mM $MgCl_2$, instead of the 5mM final concentration. The partially purified enzyme preparation (1.23µg protein/assay), which had a specific activity of 1.024 mU/mg protein and 0.37 µg protein/µl was used for the kinetic studies.

The results are shown in Figures 3.18 and Figure 3.19 as plots of activity *versus* ADP concentration. It can be seen that ADP acts as an inhibitor for all four concentrations of $MgATP^{2-}$ and R5P. In order to assess more quantitatively the effect of substrate (S) concentration on the ADP inhibition, the results are expressed as fractional inhibition (i) and are plotted against ADP concentration in the inserts of Figures 3.18 and Figure 3.19. From these plots an 'operational' inhibition constant, $i_{0.5}$ is obtained by fitting the data to the Michaelis-Menten equation by the computer programme also used for the substrate saturation experiments. The 'operational' inhibition constants are tabulated in Table 3.23.

The nature of ADP inhibition was assessed from Dixon and Cornish-Bowden plots as presented by Wharton and Eisenthal (1981). Figure 3.20

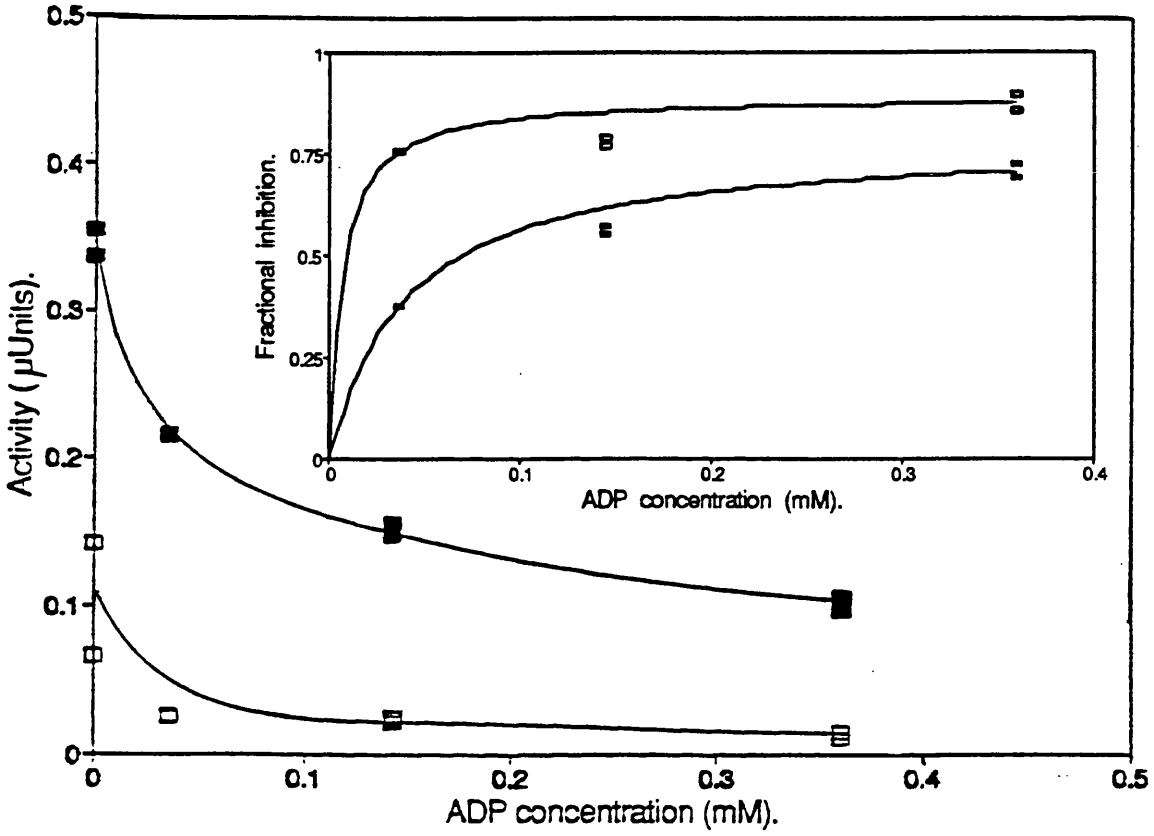


Figure 3.18. Activity against ADP concentration at fixed low R5P concentration 0.057mM and varied $MgATP^{2-}$ concentrations.

Insert shows the data plotted as fractional inhibition,i, against ADP concentration at low (□——□) and high (■——■) $MgATP^{2-}$ concentrations, at a fixed low R5P concentration of 0.057mM.

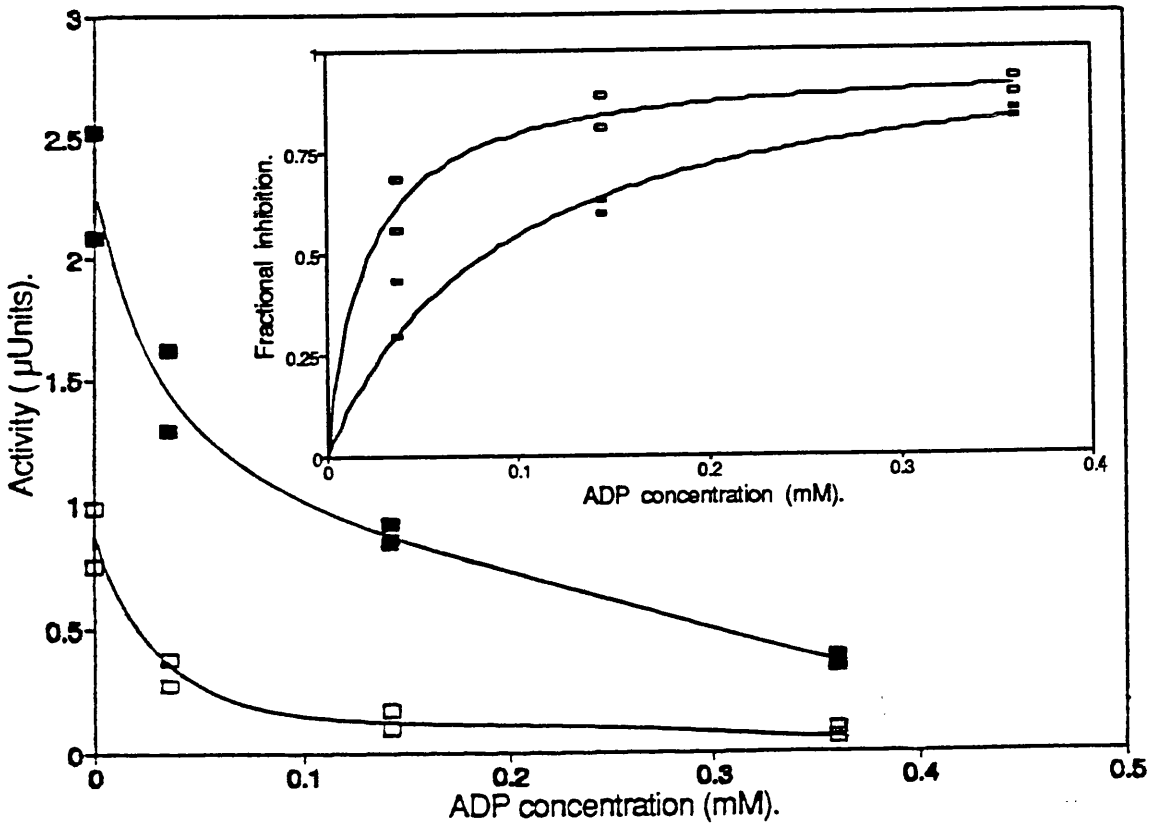


Figure 3.19. Activity against ADP concentration at fixed high R5P concentration 2.87mM and varied $MgATP^{2-}$ concentrations.
Insert shows the data plotted as fractional inhibition,i, against ADP concentration at low (□——□) and high (■——■) $MgATP^{2-}$ concentrations, at a fixed high R5P concentration of 2.87mM.

Table 3.23. Apparent kinetic parameters for the 'operational' inhibition constant of ADP for trypanosomal PRPP synthetase.

[R5P] (mM)	[$MgATP^{2-}$] (mM)	'Operational' inhibition constant (mM)
0.057	0.036	0.00646 ± 0.0144
0.057	0.360	0.03890 ± 0.0215
2.87	0.036	0.02050 ± 0.0087
2.87	0.360	0.09020 ± 0.0300

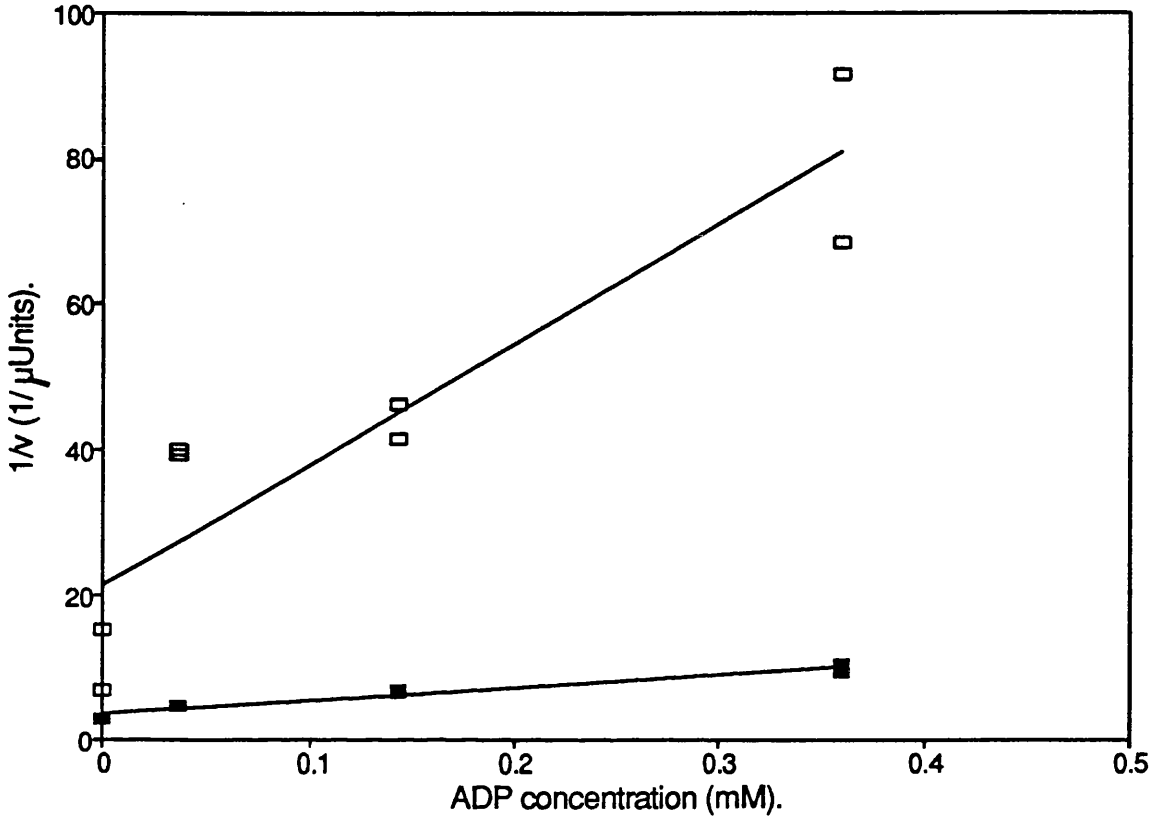


Figure 3.20. Dixon plot of 1/v against inhibitor (ADP) concentration, at a fixed low R5P concentration of 0.057mM and varying $MgATP^{2-}$ concentrations, at a total concentration of Mg^{2+} in the assay of 10mM.

□——□ represents the assay with [$MgATP^{2-}$]= 0.036mM,

■——■ represents the assay with [$MgATP^{2-}$]= 0.36mM.

and figure 3.21 are the results plotted as Dixon plots for R5P fixed at low and high concentration respectively while $MgATP^{2-}$ was varied. In both cases the lines cross in the second quadrant (above the x-axis). Similarly Figures 3.22 and 3.23 present the data as Dixon plots at fixed low and high $MgATP^{2-}$ concentrations while varying R5P. In both cases the lines cross in the third quadrant (below the x-axis). Figures 3.24 and 3.25 present the data as Cornish-Bowden plots at fixed low and high R5P concentrations and varying $MgATP^{2-}$ concentration respectively. In these cases, the lines cross in the third quadrant (below the x-axis). Similarly Figures 3.26 and 3.27 present the data as Cornish-Bowden plots at fixed low and high $MgATP^{2-}$ concentrations and varying the R5P concentration respectively. In these cases the lines cross in the second quadrant (above the x-axis).

From the Dixon and Cornish-Bowden plots it appears that ADP acts as a mixed inhibitor with respect to both substrates. The inhibitor constants Ki (for the free enzyme) and Ki′ (for the substrate-enzyme complex) were estimated using the Dixon and Cornish-Bowden plots respectively. The slopes and intercepts of the computed 'best-fit' staight lines through the data were used to calculate the inhibition constants. The x-coordinate of the intersection point of the two lines in the Dixon plot, gives the value of -Ki, while the respective point from the Cornish-Bowden plot gives the value of the -Ki′. The estimates of the inhibition constants are given in Table 3.24.

In the case of R5P, the mixed inhibition is R5P-inhibited (Ki<Ki′), while in the case of $MgATP^{2-}$ the mixed inhibition by ADP appears to be $MgATP^{2-}$-enhanced (Ki>Ki′). Further experiments need to be carried out with more R5P concentrations to clearly determine whether at low R5P concentrations the ADP inhibition is of a competitive nature or not.

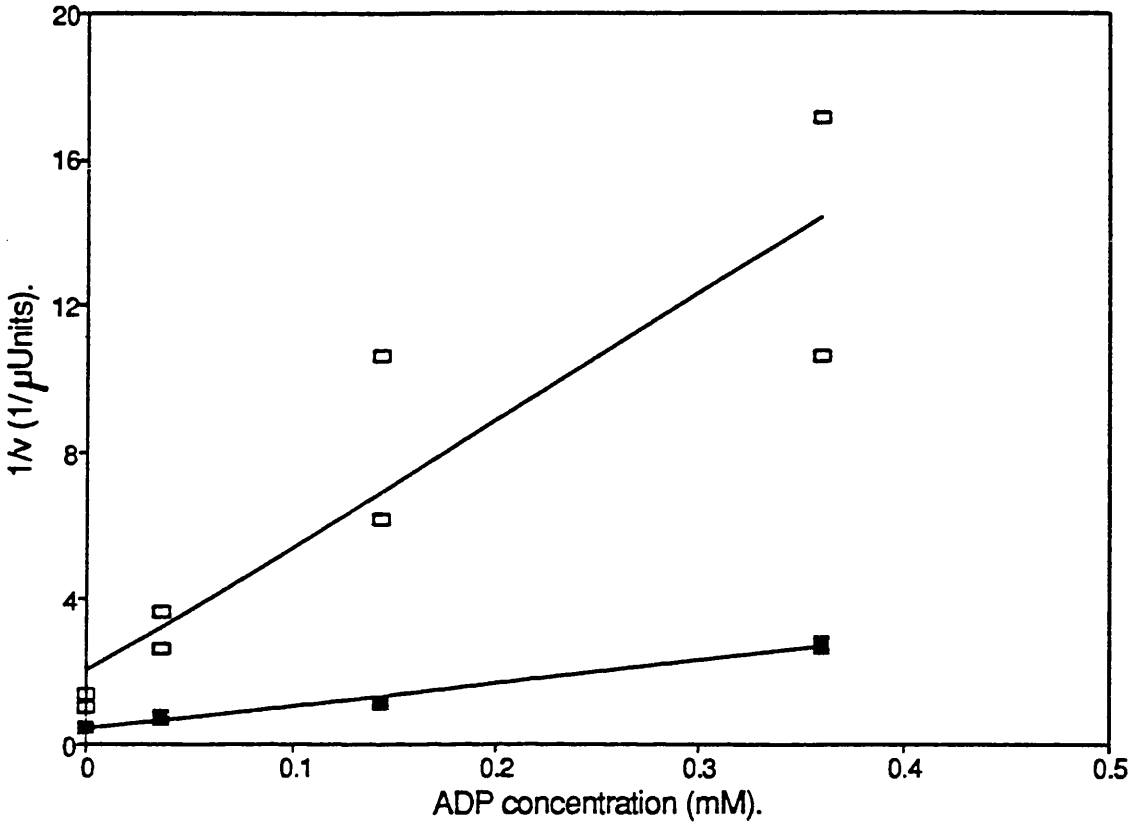


Figure 3.21. Dixon plot of 1/v against inhibitor (ADP) concentration, at a fixed high R5P concentration of 2.87mM and varying $MgATP^{2-}$ concentrations, at a total concentration of Mg^{2+} in the assay of 10mM.

□——□ represents the assay with [$MgATP^{2-}$]= 0.036mM,

■——■ represents the assay with [$MgATP^{2-}$]= 0.36mM.

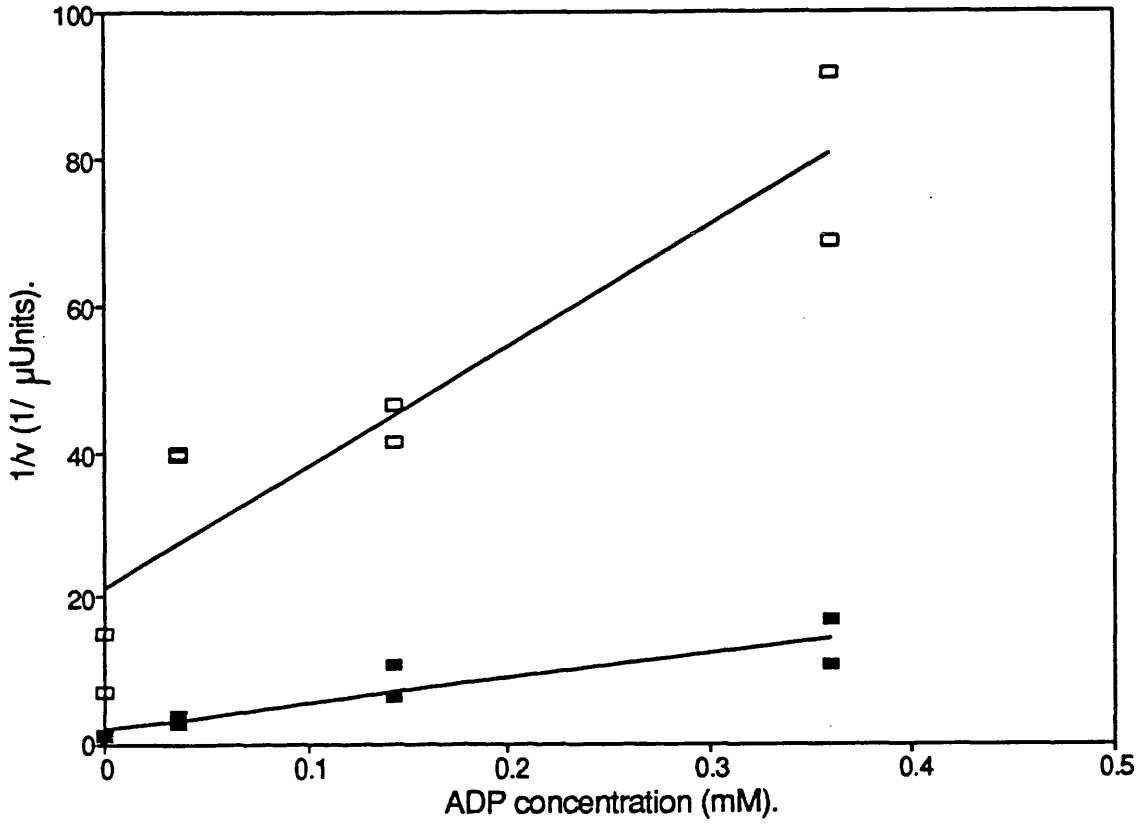


Figure 3.22. Dixon plot of 1/v against inhibitor (ADP) concentration, at a fixed low $MgATP^{2-}$ concentration of 0.036mM and varying R5P concentrations, at a total concentration of Mg^{2+} in the assay of 10mM.

□——□ represents the assay with [R5P]= 0.057mM,

■——■ represents the assay with [R5P]= 2.870mM.

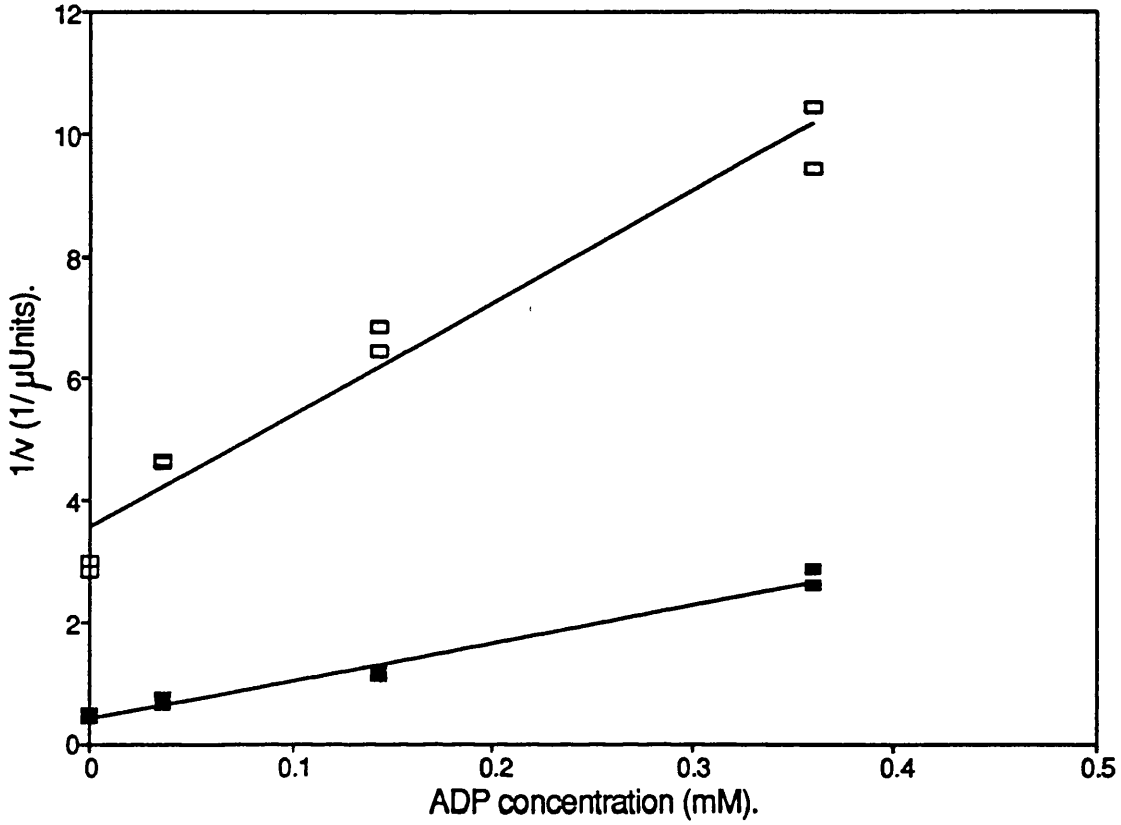


Figure 3.23. Dixon plot of 1/v against inhibitor (ADP) concentration, at a fixed high $MgATP^{2-}$ concentration of 0.36mM and varying R5P concentrations, at a total concentration of Mg^{2+} in the assay of 10mM.

□——□ represents the assay with [R5P]= 0.057mM,

■——■ represents the assay with [R5P]= 2.870mM.

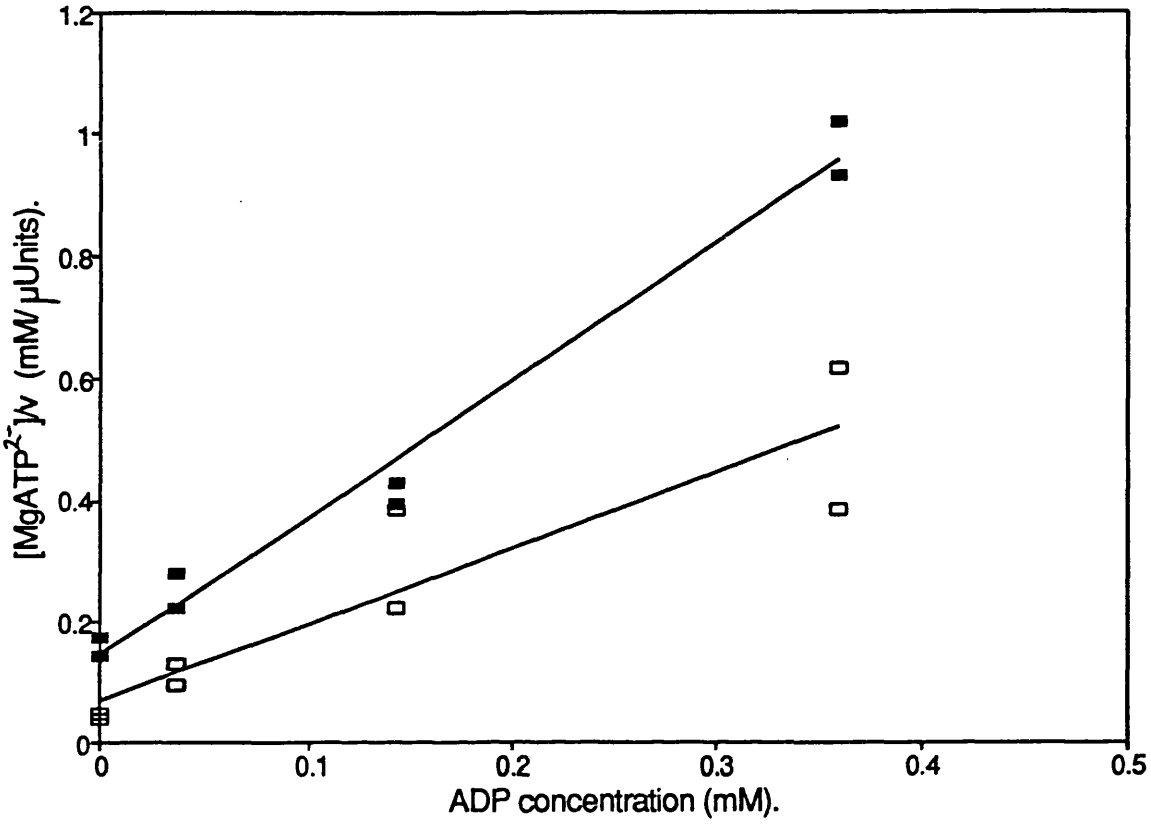


Figure 3.25. Cornish-Bowden plot of $[MgATP^{2-}]/v$ against inhibitor (ADP) concentration at fixed high R5P concentration of 2.87mM and varying $MgATP^{2-}$ concentrations, at a total concentration of Mg^{2+} in the assay of 10mM.

□——□ represents the assay with $[MgATP^{2-}]$= 0.036mM,

■——■ represents the assay with $[MgATP^{2-}]$= 0.360mM.

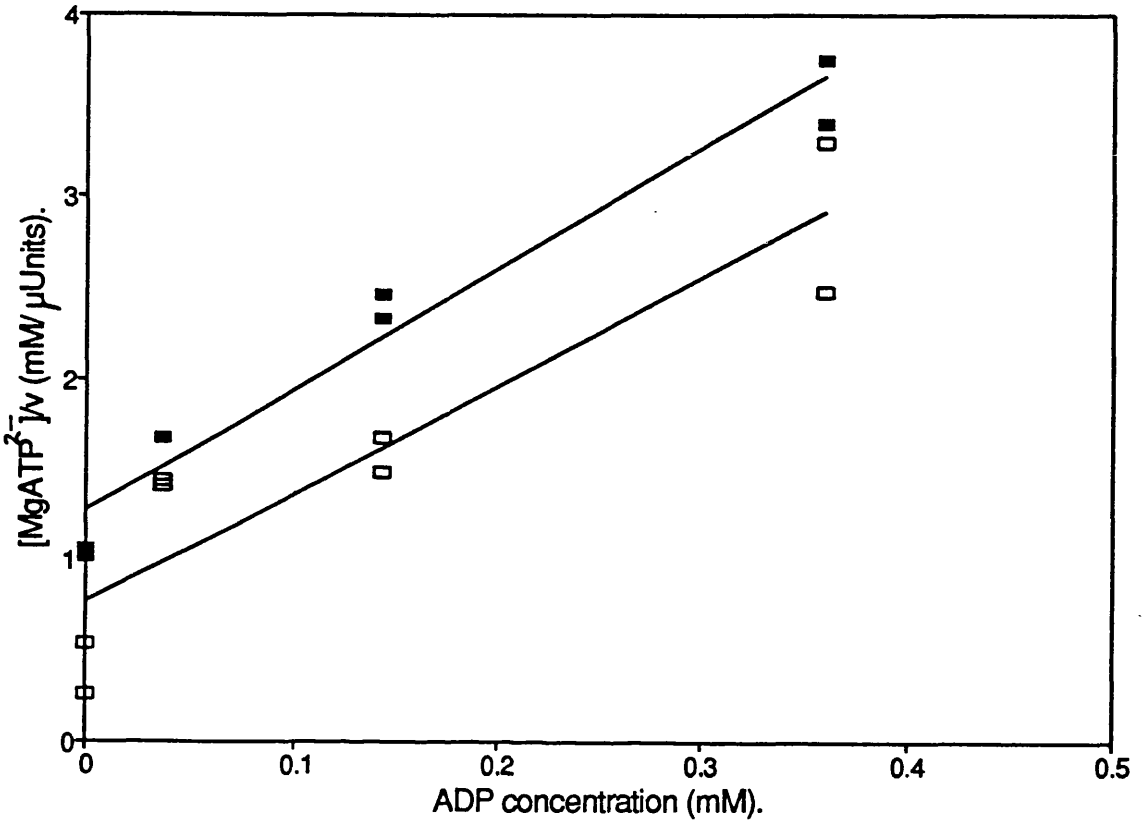


Figure 3.24. Cornish-Bowden plot of $[MgATP^{2-}]/v$ against inhibitor (ADP) concentration at fixed low R5P concentration of 0.057mM and varying $MgATP^{2-}$ concentrations, at a total concentration of Mg^{2+} in the assay of 10mM.

□——□ represents the assay with $[MgATP^{2-}]$= 0.036mM,

■——■ represents the assay with $[MgATP^{2-}]$= 0.360mM.

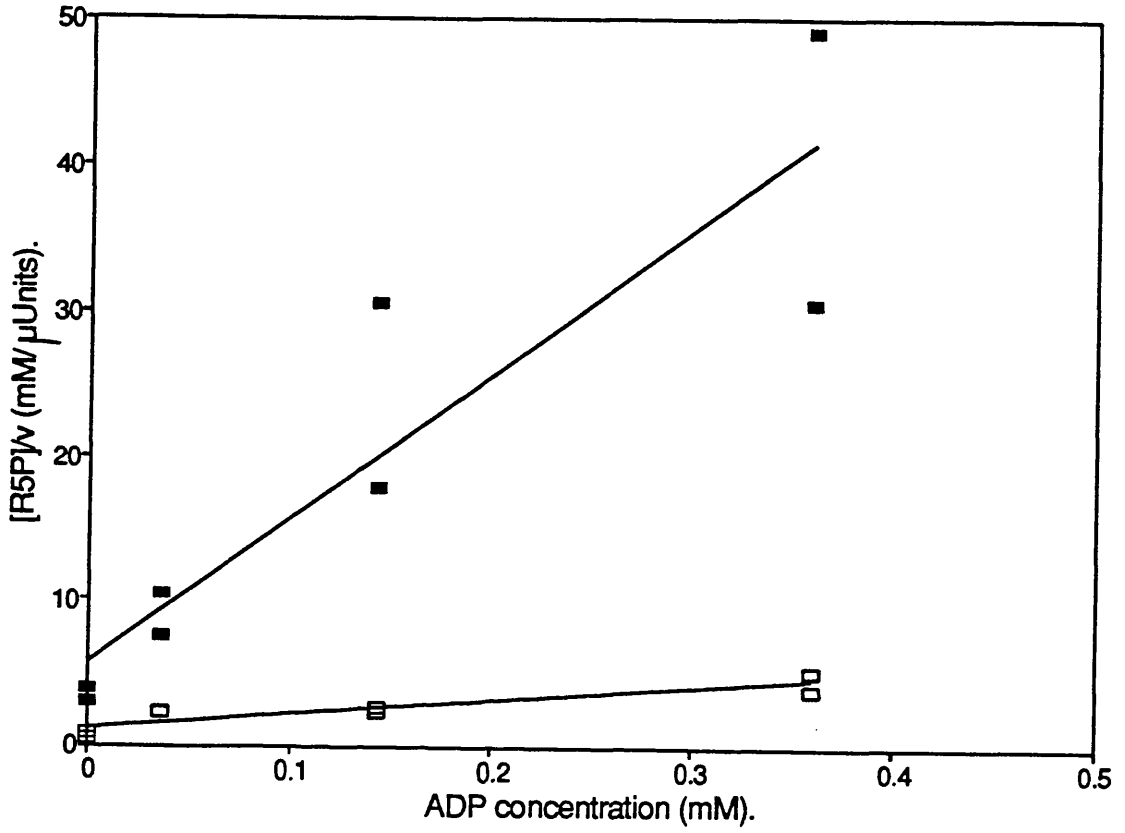


Figure 3.26. Cornish-Bowden plot of [R5P]/v against inhibitor (ADP) concentration at fixed low $MgATP^{2-}$ concentration of 0.036mM and varying R5P concentrations, at a total concentration of Mg^{2+} in the assay of 10mM.

□——□ represents the assay with [R5P]= 0.057mM,

■——■ represents the assay with [R5P]= 2.870mM.

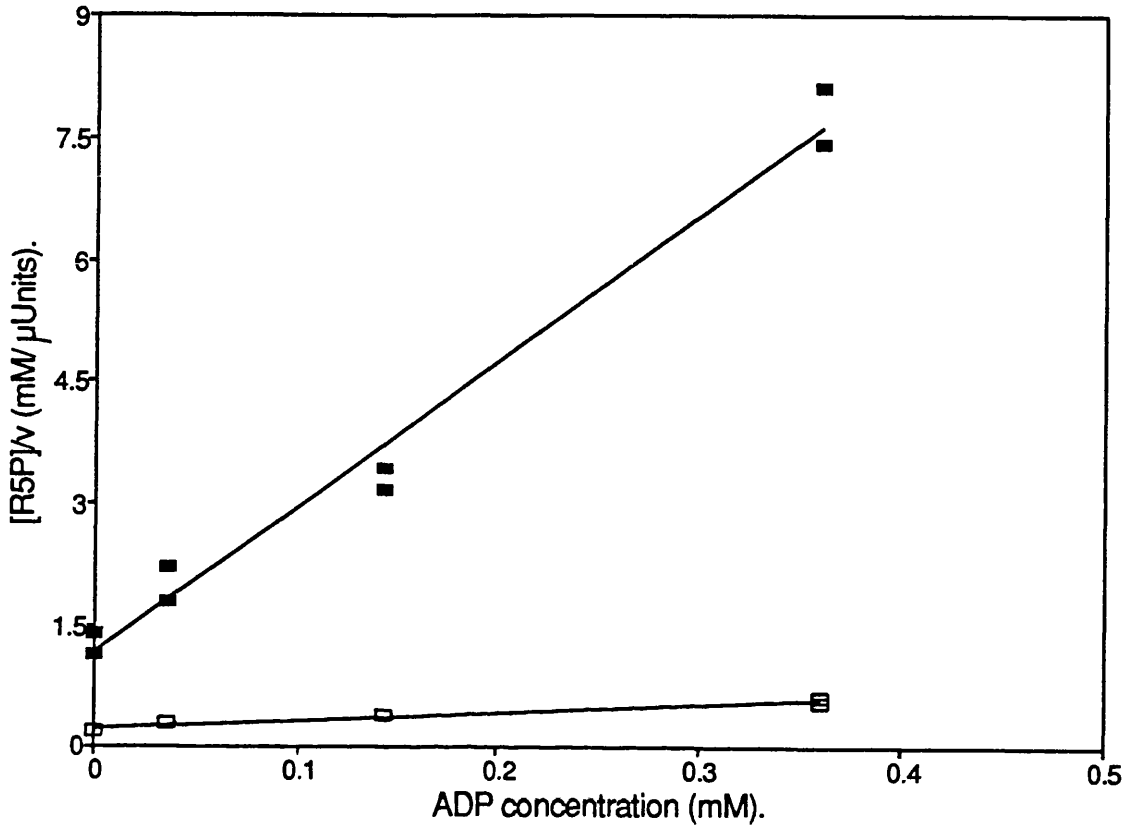


Figure 3.27. Cornish-Bowden plot of [R5P]/v against inhibitor (ADP) concentration at fixed high $MgATP^{2-}$ concentration of 0.36mM and varying R5P concentrations, at a total concentration of Mg^{2+} in the assay of 10mM.

□——□ represents the assay with [R5P]= 0.057mM,

■——■ represents the assay with [R5P]= 2.870mM.

Table 3.24. Calculated apparent kinetic parameters for the ADP inhibited trypanosomal PRPP synthetase.

[R5P] (mM)	[$MgATP^{2-}$] (mM)	Ki (mM)	Ki′ (mM)
0.057	varied	0.122 ± 0.084	0.772 ± 1.591
2.870	varied	0.056 ± 0.089	0.074 ± 0.104
varied	0.036	0.149 ± 0.098	0.050 ± 0.081
varied	0.360	0.261 ± 0.063	0.057 ± 0.0238

3.4.31. Results on localisation study of *T.b.brucei* PRPP synthetase.

Distribution patterns of marker enzymes, PRPP synthetase and protein were carried out by the group in Brussels, as previously described by Steiger *et al.* (1980). Since density boundaries are not strictly reproducible, the histograms were normalised over 15 fractions. The density scale, divided into 15 normalised fractions of identical increment, extends from 1.07 to 1.27 g/cm^3. The surface area of each histogram section is equivalent to the fractional amount of constituent present within each normalised fraction (Steiger, Opperdoes and Bontemps, 1980).

The PRPP synthetase activity was expressed in milliunits, that is the amount of enzyme catalysing the formation of 1 nmole of PRPP per minute under the standard assay conditions employed. The results, which are presented in table 3.25 are expressed as relative activity in each fraction as a percentage of the activity of fraction 14 and sent to the laboratory of origin.

The data were analysed in Brussels, using a subcellular fractionation computer programme and the results presented in Figure 3.28, where the distribution of the PRPP synthetase was compared with those of a number of marker enzymes. The abbreviations used were as follows: prot, protein; manno, α-D-mannosidase (lysosomes); AcPase, acid phosphatase (flagellar pocket); α-gluco, α-D-glucosidase (plasma membrane); ALAT, alanine aminotransferase (cytosol); HK, hexokinase (glycosomes); ICDH-P, NADP-linked isocitrate dehydrogenase (mitochondrion); 3-AMPase, 3'-nucleotidase (plasma membrane); PRPP, phosphoribosylpyrophosphate synthetase. The recovery (R) is indicated within parenthesis. In the case of PRPP synthetase, the recovery is 89.7%. PRPP synthetase behaves as a soluble enzyme. The peak of PRPP synthetase activity is extremely sharp and the fraction with the highest activity was at a sucrose density of around 1.11 to 1.13 g cm^{-3}.

From figure 3.28 it is clear that PRPP synthetase sediments much faster than alanine aminotransferase or the soluble components of α-D-glucosidase or 3'-nucleotidase. As discussed by Dr.F.R. Opperdoes (personal communication),

Table 3.25. Phosphoribosylpyrophosphate synthetase (EC 2.7.6.1) activity in subcellular fractions of bloodstream form of *T.brucei*.

Gradient TR 21A (STE-GRADIENT 211083)

Fraction	Activity * (munits ml^{-1})	Relative activity **
1	0.0256	0.84
2	0.0190	0.62
3	0.0563	1.85
4	0.0495	1.62
5	0.0342	1.12
6	0.0335	1.10
7	0.0417	1.37
8	1.0163	33.31
9	4.9770	163.11
10	0.5967	19.55
11	0.1076	3.53
12	0.0530	1.74
13	0.0253	0.83
14	3.0514	100.00

The activity of PRPP synthetase in each fraction where,

* milliunits are nmoles of PRPP produced per minute.

** relative activity is expressed as a percentage of the activity of fraction 14.

The assay contained twice the units of HGPRTase (4munits/assay) and 12μM [^{14}C]hypoxanthine in the assay. All the other variables of the assay were the same as in the standard assay.

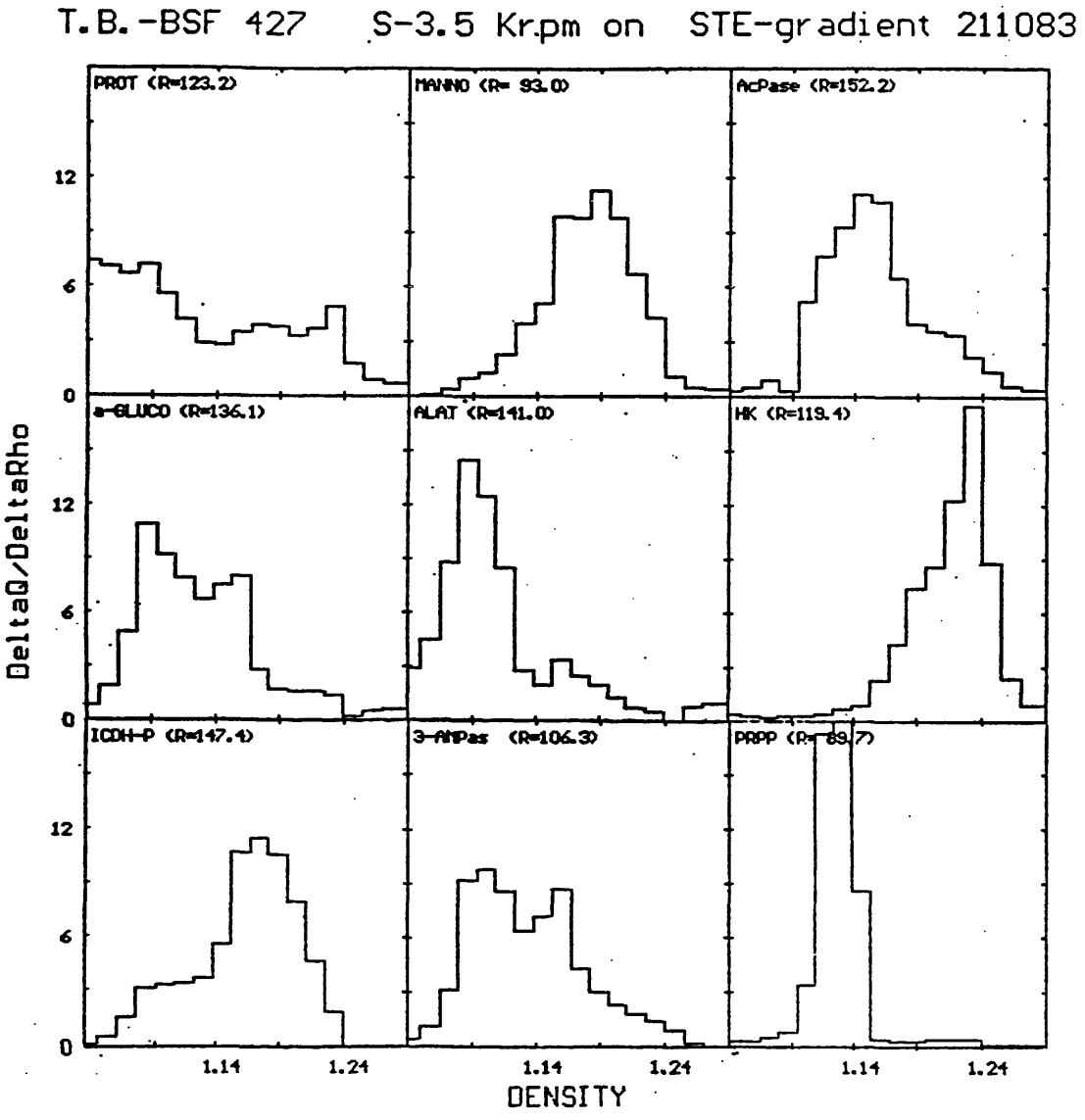


Figure 3.28. Isopycnic centrifugation in sucrose of a large-granule fraction (65.3mg protein) from *T.b.brucei*. The percentage of recovery (sum of the activity of all fractions with respect to the activity of the layered sample) for each protein is given within parentheses. Frequency, on the ordinate, is defined as the percentage of recovered activity in a fraction divided by the density range covered by the same fraction. The results are duplicates of a single experiment (See text for abbreviations).

this is a peculiar behaviour for a soluble enzyme unless it has a very high molecular weight due to aggregation. An alternative explanation offered, would be that the enzyme was associated with membrane vesicles equilibrating at a density of around 1.11 to 1.13 g cm^{-3}. As pointed out, membrane vesicles normally give a very broad distribution due to their heterogeneity, rendering this explanation less likely. It is reported in the literature that PRPP synthetase from other sources, such as, rat liver (Roth *et al.*, 1974), *Salmonella typhimurium* (Switzer, 1969) and human erythrocytes (Fox and Kelley, 1971), exists as a low solubility enzyme with a large molecular weight. The size of the respective enzyme from the bloodstream form of *T.brucei* was investigated both in the crude extract and in the partially purified preparation (see following section).

3.4.32. Gel filtration of PRPP synthetase from *T.brucei.*

Calibration of Superdex S-200 column.

Figure 3.29 shows the graph of elution volume plotted against the log molecular weight of standard proteins on the Superdex S-200 column which was equilibrated with 50mM potassium phosphate, pH 7.6, 6mM magnesium chloride, 1mM EDTA (disodium salt) and 2.5mM mercaptoethanol.

Partially purified PRPP synthetase from *T.brucei.*

In the partially purified enzyme preparation, PRPP synthetase activity eluted from Superdex S-200 column after 59ml (figure 3.30). From figure 3.29 this elution volume corresponds to a molecular weight of about 193,000.

The specific activity of the partially purified heat treated enzyme preparation was 0.421 munits/mg protein. The enzyme recovery in fraction 59 was 4% of total units loaded but the specific activity enrichment was 18.9x to 7.957 munits/mg protein.

Crude homogenate of *T.brucei.*

The elution profile of PRPP synthetase activity from Superdex S-200 gel filtration column in a crude *T.b.brucei* homogenate is presented in Figure 3.31.

With crude homogenate the PRPP synthetase activity eluted as a large peak in the void volume of the column. The exclusion limit of the column was 600,000. The Dextran blue 2000 eluted after 38ml, the void volume of the column. The molecular weight of the PRPP synthetase in the crude homogenate was estimated to be greater than 600,000.

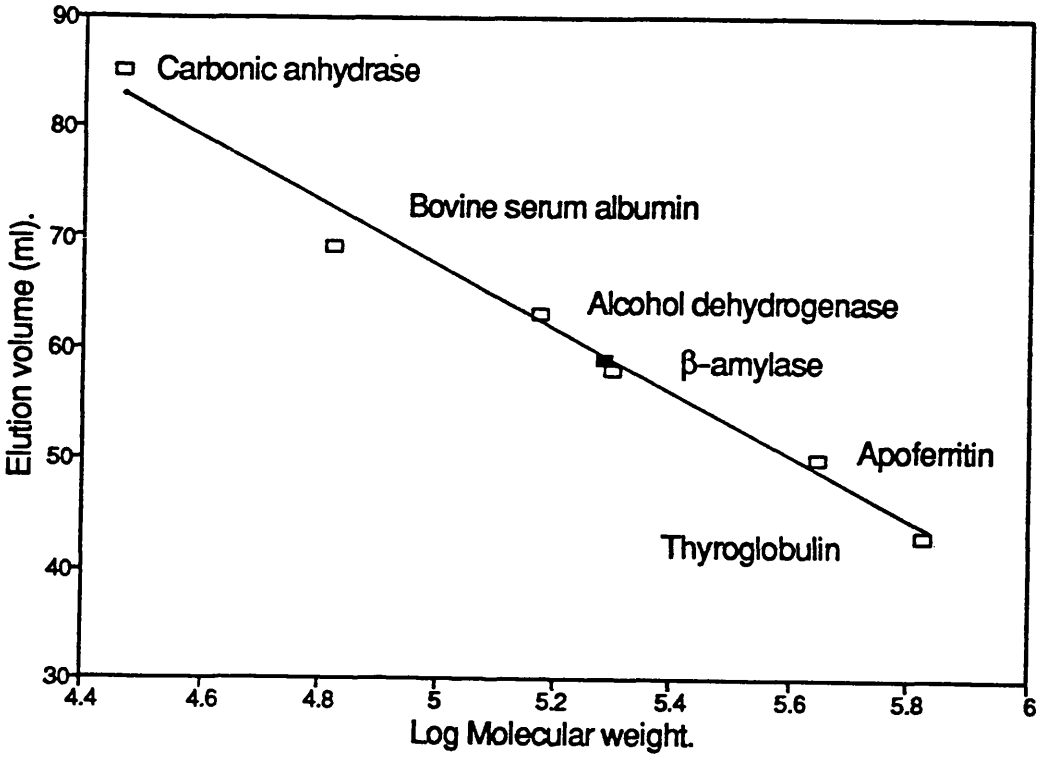


Figure 3.29. Gel filtration of PRPP synthetase from *T.brucei*.

Calibration of Superdex S-200 (16/60) gel filtration column with known molecular weight marker proteins. The equilibration and elution buffer was 50mM potassium phosphate, pH 7.6, 6mM magnesium chloride, 1mM EDTA (disodium salt) and 2.5mM mercaptoethanol. The least square regression indicates a molecular weight of about 193,000 for partially purified *T.brucei* PRPP synthetase preparation (position marked with a filled square).

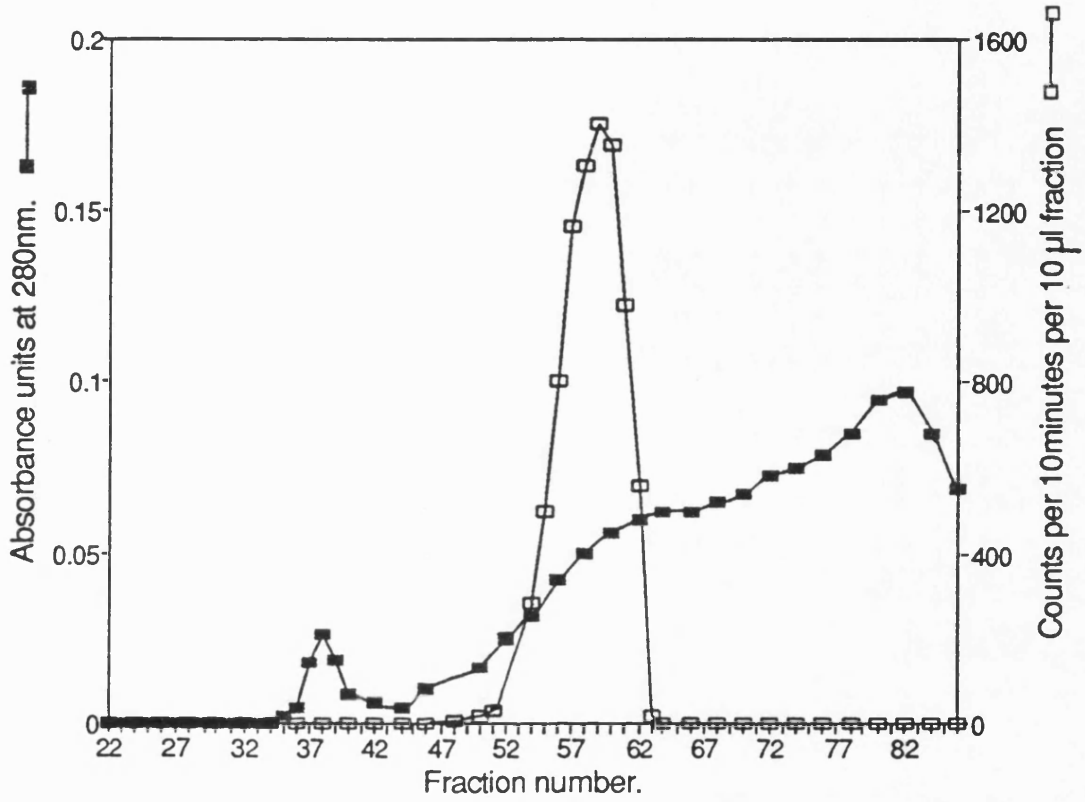


Figure 3.30. Elution of PRPP synthetase activity from Superdex S-200 gel filtration column using a partially purified enzyme preparation.

The equilibration and elution buffer was 50mM potassium phosphate, pH 7.6, 6mM magnesium chloride, 1mM EDTA (disodium salt) and 2.5mM mercaptoethanol. Fraction volume was 1ml. The enzyme activity is expressed as counts per 10 minutes per 10µl of fraction assayed with the standard method.

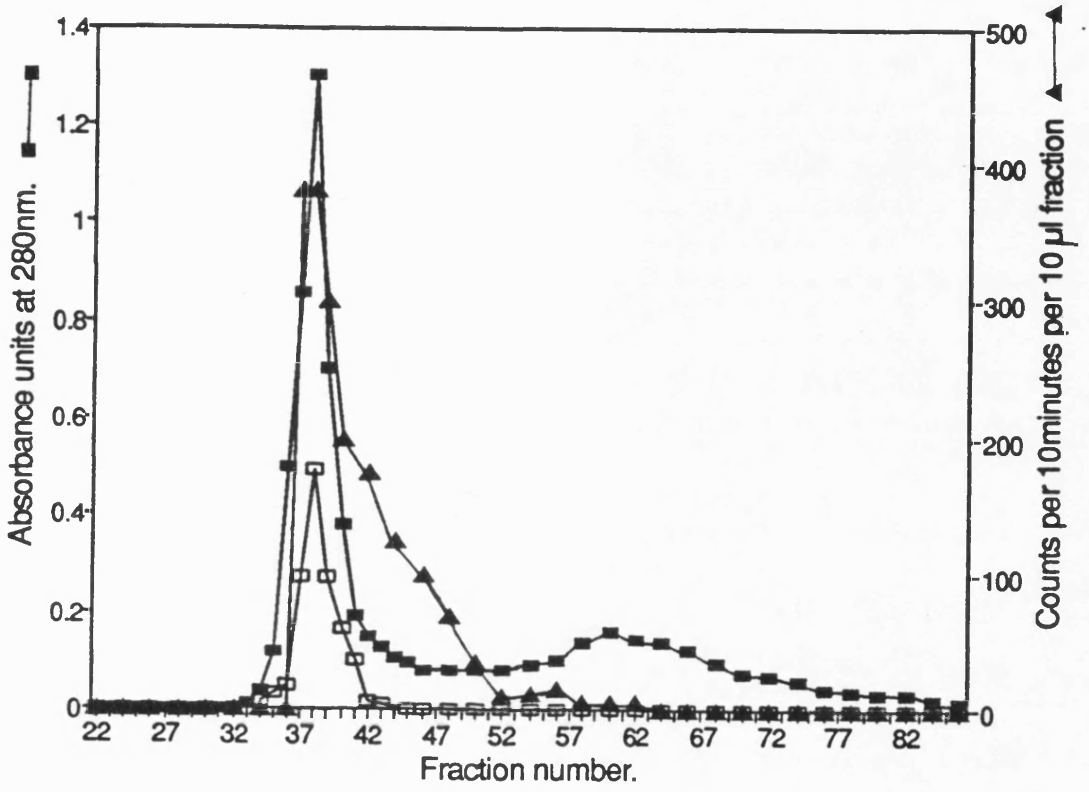


Figure 3.31. Elution of PRPP synthetase activity from Superdex S-200 gel filtration column in a crude *T.b.brucei* homogenate.

Blue dextran elution is marked with □——□ . The equilibration and elution buffer was 25mM Tris-HCl, pH 7.2, 1mM EDTA (disodium salt) and 0.25 M sucrose. The fraction volume was 1ml. The enzyme activity was expressed as counts per 10 minutes per 10µl of fraction assayed with the standard method. Both dextran blue and PRPP synthetase eluted in the void volume of the colume which is 38ml. The molecular weight of the enzyme was therefore greater than the column exclusion limit of 600,000.

3.5. Discussion.

From the data described in Chapter 2 it was confirmed that the bloodstream forms of *T.b.brucei* converted a small, but significant proportion of the metabolised glucose to C_5 carbohydrate which was incorporated into the RNA of the cells. PRPP synthetase is the sole enzyme responsible for formation of PRPP which gets converted into nucleotide building blocks through the action of phosphoribosyltransferases. A number of phosphoribosyltransferases had been detected and quantitated in crude extracts of *T.b.brucei* by other workers (Gutteridge and Davies, 1982 and Davies *et al.*, 1983). The detection of the sole enzyme responsible for the formation of the key metabolite PRPP was therefore pursued beyond the initial failure to detect it by the orotate removal assay. The sensitivity of the orotate removal assay was inadequate for detection of the enzyme from the trypanosomes. Equally limited was the use of the ^{32}P transfer assay due to the inadequate purification of the enzyme from *T.b.brucei*. As it was shown in the pentose phosphate specificity experiments the partially purified trypanosomal PRPP synthetase preparation contained other contaminating enzymatic activities that did not allow the use of the ^{32}P transfer assay. The method was tried on the partially purified enzyme preparation but found to yield very high blanks that made the assay impossible to use. This assay is ideal because it is the only direct assay to measure PRPP synthetase and because it is fast and very accurate, it is preferred for kinetic assays of highly purified enzyme preparations. Its drawback is the requirement for pure enzyme preparations free from ATPase-like activities such as ribose-5-phosphate kinase, ribulose-5-phosphate kinase, and ribokinase.

It was found that the coupling of PRPP synthetase to HGPRTase resulted in a useful and reliable method for assaying the activity of PRPP synthetase from trypanosomes. The purification protocol resembled that of the same enzyme from different sources but with the inherent problem of very low specific activity. As for the same enzyme from rat liver the extent of purification depended on the specific activity of the crude extract (Roth *et al.*, 1974). The yield of partially

purified PRPP synthetase was adequate for a simple kinetic study and for comparison of some of the properties of the trypanosomal enzyme to those of the enzyme from other sources.

The large number of potential inhibitors of PRPP synthetase from other sources (Fox and Kelley, 1971) makes speculation regarding the endogeneous inhibitor present in the crude extract difficult. It must be of small size since it was removable by dialysis and gel filtration by passing the extract through a Sephadex G-25 column. The endogeneous inhibitor may have been ADP, since this was found to be inhibitory to trypanosomal PRPP synthetase. Further work would be required to isolate and characterise the endogeneous substance of inhibitory action on trypanosomal PRPP synthetase.

Inorganic phosphate activates all known PRPP synthetases including the trypanosomal one. Inorganic phosphate activated the trypanosomal PRPP synthetase but was not an absolute requirement for its stability since in its absence the enzyme remained stable. This is in contrast to the situation with human erythrocyte and the *S.typhimurium* PRPP synthetases which have an absolute requirement for phosphate for both stability and catalytic activity (Fox and Kelley, 1971; Switzer, 1969). However, the rate of decay of activity of PRPP synthetase on storage at 0° and 37°C was unaffected by Pi. The trypanosomal PRPP synthetase is similar to the rat liver enzyme (Roth *et al.*, 1974), in not requiring phosphate for stability, as shown by the failure of phosphate-free buffer to inactivate the enzyme.

The rat liver enzyme has been found to be stabilized by albumin (50μg/ml), EDTA (1mM), or dithiothreitol (1mM) and loses activity when assayed at low protein concentration (Roth *et al.*, 1978). Iodoacetamide and *p*-chloromercuribenzoate abolish the effect of these compounds, suggesting that stabilisation of the enzyme is due to the protection of an essential sulphydryl group on the enzyme (Roth and Deuel, 1974). Stabilisation of PRPP synthetase by EDTA could result from chelation of contaminating heavy metals present during the assay.

Fox and Kelley (1971) showed that ATP (0.3mM) and 6mM $MgCl_2$ stabilised the human erythrocyte enzyme at -70°, 4°, and 60°C. These substances also partially protected the enzyme from inactivation by dithiothreitol at -70°C. $MgATP^{2-}$, which is the true substrate for the trypanosomal PRPP synthetase, was found to be highly inhibitory at concentrations above about 0.36mM.

The substrate specificity of trypanosomal PRPP synthetase was strict as would have been expected for a tightly regulated enzyme that supplies the cell with such a key metabolite as PRPP. Although there was no substantial activity with any of the nucleotide triphosphates or pentose phosphate species other than R5P, the blank was as high as the test in the ITP experiment. This could be due to the presence of ITP hydrolysis products which equilibrated with the radioactive IMP pool or which were generated by some contaminating enzyme activities in the partially purified preparation used. Because the test was as high as the blank, this was not due to net PRPP synthesis, since the blank contains no R5P which is required for PRPP synthetase activity.

T.b.brucei PRPP synthetase, like the rat liver (Roth *et al.*, 1974), *S.typhimurium* (Switzer, 1969) and human erythrocyte (Fox and Kelley, 1971) PRPP synthetase has strict structural requirements for the pyrophosphoryl donor. The relative unimportance for the 2-hydroxyl position of the ribose moiety of the purine nucleotides, was demonstrated in the similar activities with ATP and dATP as substrates.

The enzyme preparation utilised ribulose-5-phosphate with a similar rate as it utilised ribose-5-phosphate, suggesting the ribulose-5-phosphate could have been a substrate for PRPP synthetase or that it was converted to ribose-5-phosphate first. The first suggestion involves the formation of PPRuP which is not a substrate for HGPRTase, which has a strict substrate specificity for PRPP (Flaks, 1963a). The second explanation is more likely especially as D-ribose-5-phosphate ketol-isomerase (EC 5.3.1.6) activity may have co-purified with PRPP synthetase. As reported by Cronin *et al.* (1989), bloodstream *T.brucei* forms have D-ribose-5-phosphate ketol-isomerase activity with specific activity in a crude

extract of 144 nmoles/min/mg protein. This specific activity is much higher than that of PRPP synthetase, indicating that ribose-5-phosphate derived from ribulose-5-phosphate is supplying the PRPP synthetase and that ribose-5-phosphate formation is not rate limiting. The reaction continues as ribose-5-phosphate continues to be available to PRPP synthetase in the 30 minute incubation. From the specific activity of 144 nmoles/minute/mg protein it can be estimated that the partially purified PRPP synthetase preparation contained 1.06% of protein as D-ribose-5-phosphate ketol-isomerase.

Ribose-5-phosphate is the preferred and possibly the only pentose-phosphate substrate for trypanosomal PRPP synthetase. The lack of pure PRPP synthetase preparation makes it impossible to conclude whether the activity observed with ribulose-5-phosphate is due to PRPP synthetase or D-ribose-5-phosphate ketol-isomerase activity, which remained in the partially purified PRPP synthetase preparation.

Hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) from Brewer's yeast, which was the enzyme supplied by Sigma, has a specific requirement for PRPP which cannot be replaced with ATP, ribose-1,5-diphosphate, ribose-5-phosphate, ribose-1-phosphate or combinations of the latter two substrates with ATP (Flaks, 1963b). Because of the indirect assay for PRPP synthetase activity used, involving the coupling to HGPRTase activity, the mechanism of PRPP synthetase with pentose phosphate substrate, would have to be such that PRPP is always produced, if the activity is to be so measured.

It is therefore likely that the partially purified trypanosomal PRPP synthetase preparation has both D-ribose-5-phosphate ketol-isomerase and phosphoribomutase contaminating activities, since incubations with ribulose-5-phosphate as well as with ribose-1-phosphate resulted in PRPP synthetase activity.

PRPP synthetases from *Salmonella typhimurium* (Switzer and Gibson, 1978) and from rat liver (Roth *et al.*, 1974) have a strict specificity for ribose-5-phosphate. Purified human erythrocyte PRPP synthetase has been reported to

exhibit 34% activity with ribulose-5-phosphate as substrate compared to activity with ribose-5-phosphate (Fox and Kelley, 1971). Because of possible contamination of commercial ribulose-5-phosphate preparations with ribose-5-phosphate, the activity may not reflect the formation of PPRuP (Roth *et al.*, 1974). It is most likely that trypanosomal PRPP synthetase shares in the strict substrate specificity.

It is possible that the apparent activity with ribose-1-phosphate could have arisen from the conversion of ribose-1-phosphate and [^{14}C] hypoxanthine to [^{14}C] inosine, by the action of purine-nucleoside phosphorylase (EC 2.4.2.1), followed by nucleoside kinase to [^{14}C] IMP.

It is unlikely that PRPP synthetase can utilise ribose-1-phosphate as a substrate, although it would seem advantageous to the parasite if it could use (recycle) the ribose-1-phosphate produced by nucleoside phosphorylases to form nucleotides through PRPP synthetase. Phosphoribomutase activity is distinct from phosphoglucomutase and exists in many cells such as Baker's and Brewer's yeast, uterine muscle (rabbit, bovine, human) and in human blood (Guarino and Sable, 1956), and might also exist in trypanosomes. It would be interesting to find out if such activity exists in *T.brucei.*

Unlike PRPP synthetase of human erythrocytes (Fox and Kelley, 1971), Ehrlich ascites tumor cells (Wong and Murray, 1969) and the *S.typhimurium* enzyme (Switzer, 1969), the *T.b.brucei* enzyme showed activity with calcium. In the copper system, there was a precipitate formed. The activity with Cu^{2+} was as high as for the blank and very high for test up to 200% of that with Mg^{2+} alone.

For the *Salmonella* enzyme, Mg^{2+} is required for formation of the bidentate $MgATP^{2-}$ complex, which serves as the substrate for the enzyme and for the true product MgPRPP (Li *et al.*, 1978). Excess Mg^{2+} is also required for binding to the subunits of PRPP synthetase and activating the enzyme when $MgATP^{2-}$ is available. For the trypanosomal PRPP synthetase excess magnesium over that of ATP was found to be a competitive inhibitor with respect to $MgATP^{2-}$, suggesting the binding of magnesium at the active site of PRPP

synthetase at the binding site of $MgATP^{2-}$. Because of the coupling assay used, the magnesium concentration was kept at 5mM in the standard assay since HGPRTase activity was enhanced by free magnesium.

PRPP synthetase from *T.brucei* showed a high affinities for its substrates, R5P and $MgATP^{2-}$ with apparent Km of 27μM and 63μM respectively. The Km values for the trypanosomal PRPP synthetase are comparable to the Km values for this enzyme from other sources as shown in Table 3.26. The Km values for R5P and $MgATP^{2-}$ of the trypanosomal enzyme resemble those of the Ehrlich ascites tumor cells and the human erythrocytes.

The trypanosomal enzyme showed high substrate inhibition by $MgATP^{2-}$ as the *Salmonella* enzyme, but with higher Ki. High substrate inhibition by $MgATP^{2-}$ is also reported for the pigeon liver PRPP synthetase where ATP levels greater than 2mM (Flaks, 1963b). The falling off of the rate at $MgATP^{2-}$ concentrations greater than 0.35mM thus indicates that trypanosomal PRPP synthetase has a sensitivity to substrate concentration not shown by PRPP synthetase from other sources. Whether this represents a control feature of this enzyme in the metabolic pathway leading to RNA ribose must await future studies. These studies would have to involve a detailed analysis of the subcellular concentrations of ATP, Mg^{2+} and R5P, and studies of $MgATP^{2-}$ and R5P kinetics.

The most potent inhibitor of human, Ehrlich ascites tumor cell, and rat liver PRPP synthetases is ADP, a competitive inhibitor of the enzyme with respect to $MgATP^{2-}$ (Fox and Kelley, 1972; Wong and Murray, 1969; and Roth and Deuel, 1974). ADP was found to be an inhibitor of trypanosomal PRPP synthetase with a Ki of about 0.055mM. Although the inhibition seems to show trends of mixed inhibition in nature, it appears from the inhibition constants obtained that within experimental error the inhibition may be competitive with respect to both R5P and $MgATP^{2-}$. Further work is required to give a better understanding of the system. The human erythrocyte enzyme shows an inhibition constant (Ki) for ADP of 10^{-5} M, a value well below the intracellular ADP

Enzyme source	Km for $MgATP^{2-}$	Km for R5P	Additional comments.
T.brucei bloodstream forms (This work)	63 μM	27 μM	Substrate inhibition by $MgATP^{2-}$ with Ki 250 μM
Human erythrocytes (Fox and Kelley, 1971)	14 μM	33 μM	
S. typhimurium (Switzer, 1971)	46 μM	160 μM	Substrate inhibition by $MgATP^{2-}$ with Ki 64 μM
Ehrlich Ascites tumor cells (Wong and Murray, 1969)	60 μM	50 μM	
Rat liver (Roth *et al.*, 1974)	220 μM	290 μM	Substrate inhibition at R5P concentration > 1.5 mM

Table 3.26. Kinetic parameters of PRPP synthetase from various sources.

concentration for human erythrocytes (Fox and Kelley, 1972). This finding suggests a physiological role for the competitive interaction of adenylates in controlling enzyme activity and supports the proposal of Atkinson and Fall (1967). Atkinson and Fall (1967) proposed that the potent inhibition of *E.coli* PRPP synthetase by ADP may be part of a general ATP conservation system that causes the rate of expenditure of ATP to be curtailed when the "energy charge" of the cell decreases slightly; and that such ATP conservation control may be superposed on product negative feedback regulation of PRPP synthetase. The "energy charge" of the adenylate system, defined as (ATP + 1/2 ADP)/(AMP + ADP + ATP), has been proposed by Atkinson and Walton (1967) as a fundamental metabolic control parameter.

"Energy charge" studies would have to be acompanied by compartmentalisation studies because the energy charge of the glycosomes would be expected to be markedly different to the energy charge of the cytosol.

The detection of PRPP synthetase in combination with the incorporation of glucose into the ribose moiety of RNA, gives good evidence for the formation of PRPP *in vivo*, presumably from R5P precursor. The R5P precursor may not be exclusively supplied from an active pentose phosphate pathway, but certainly a fraction of it is.

Cronin *et al.* (1989) mentioned that all the enzymes of the pentose phosphate pathway in bloodstream forms of *T.brucei* are present in the cytosol, although no direct evidence for this was given. The behaviour of PRPP synthetase as a soluble enzyme is consistent with the results of Cronin *et al.* (1989), in view of R5P being the substrate of the PRPP synthetase reaction as well as the end-product of the classical oxidative pentose phosphate pathway.

Trypanosomes depend on active salvage and interconversion pathways for both purine bases and nucleosides since they are incapable of *de novo* purine synthesis. Incorporation of purine bases and nucleosides has been demonstrated in mammalian host forms of *T.b.brucei* (James and Born, 1980). Three phosphoribosyltransferase activities with fairly high levels of activity

(0.6 - 5.6 nmole/min/mg protein ; for *T.brucei* trypomastigotes), capable of salvaging adenine, hypoxanthine and guanine were reported (Davies *et al.*, 1983). Higher rates were found for these activities than for the purine nucleoside kinases(0.045 - 0.4 nmole/min/mg protein). The same workers failed to detect inosine phosphorylase activity and suggested that hypoxanthine salvage occurs only *via* the phosphoribosyltransferase route, while there were detectable adenosine and guanosine phosphorylase activities (2.9 and 0.36 nmole/min/mg protein, respectively). Purine metabolism of the bloodstream forms of African trypanosomes is still poorly understood.

Gutteridge and Davies (1982) showed that purine phosphoribosyl-transferase activity in the culture epimastigote forms of *T.cruzi* involves two separate enzyme proteins: one, active with adenine as substrate, occurs in the cytosol; the other, active with both hypoxanthine and guanine, occurs in the glycosomes. The activities with hypoxanthine and probably guanine were found to be particulate but not latent. Isopycnic centrifugation in sucrose gradients confirmed the particulate nature of the activities with hypoxanthine and guanine and showed that they banded in the density range 1.23 - 1.25 g/cm^3, identical to that of glycolytic enzymes such as hexokinase and phosphofructokinase (Gutteridge and Davies, 1982). Whether HGPRTase activity is located on the outside of or within the glycosome is not clear. The apparent lack of latency compared with glycolytic enzymes is not significant since overall rates of reaction are lower and therefore it is unlikely that penetration of substrates would be limiting as it is with glycosomal enzymes. If it occurs within the glycosome, it is most likely to occur in the lumen or loosely attached to the membrane since it can easily be solubilised by freezing and thawing (Hammond and Gutteridge, 1980).

Hammond *et al.* (1981) showed that the last two enzymes of UMP synthesis *de novo*, OPRTase and ODCase, co-sedimented with glycosomal-(microbody-)marker enzymes such as hexokinase (see figure 1.13 in the introduction section).

The relevance of the glycosomal location of purine and pyrimidine phosphoribosyltransferases (PRTases) is unclear, but the common requirement of the enzymes for PRPP suggests that glycosomes may have a transport system for this compound or that the purine PRTases may be located on the surface of the organelle as has been suggested for orotate PRTase (Hammond and Gutteridge, 1983). From similar studies on the purine metabolising enzymes of promastigote forms of *L.m.mexicana* (Hassan *et al.*, 1985) it is suggested that channelling of purines, nucleosides and nucleotides between subcellular compartments is an integral part of purine metabolism in *T.b.brucei*, *T.cruzi* and *L.m.mexicana*.

Another possibility would be that if cytosolic R5P is the substrate for at least part of PRPP synthetase activity, and if glucose is the source for this R5P then this may suggest a source of phosphorylated C_6 intermedeates to serve as substrate for the pentose phosphate enzymes in the cytosol which in turn suggests hexokinase activity in the cytosol as well as glycosome, or transport of phosphorylated hexose from the glycosome to the cytosol.

Initially, partially purified enzyme preparation of PRPP synthetase from the bloodstream form of *T.brucei*, was found to have a molecular weight of about 193,000.

From the localisation study, *T.brucei* PRPP synthetase behaves as a soluble enzyme. However PRPP synthetase sediments much faster than alanine aminotransferase or the soluble components of α-D-glucosidase or 3'-nucleotidase. As discussed by Dr.F.R. Opperdoes (personal communication), this is a peculiar behaviour for a soluble enzyme unless it has a very high molecular weight due to aggregation.

This was found to be the case in subsequent gel filtration experiments with *T.brucei* crude extract, prepared in the same way as for the localisation study, which resulted in PRPP synthetase eluting in the void volume with molecular weight greater than 600,000. This finding explaines the sedimentation

behaviour of the enzyme. It also indicates another similarity of *T.brucei* PRPP synthetase to PRPP synthetase from other sources, such as, rat liver (Roth *et al.*, 1974), *Salmonella typhimurium* (Switzer, 1969) and human erythrocytes (Fox and Kelley, 1971), which is reported in the literature to exist as a low solubility enzyme with a large molecular weight.

PRPP synthetase from all the sources studied this far, appears as a multimeric enzyme that exists in various states of subunit aggregation.Results from gel filtration and sedimentation behaviour in localisation studies showed that PRPP synthetase from *T.brucei* also exists in more than one state of aggregation. In the crude homogenate which was prepared in the absence of magnesium, the enzyme exists as an aggregate of high molecular weight. During the partial purification, the aggregate dissociates to an enzyme species of about 193,000 molecular weight which is catalytically active.

The subunit structure of human PRPP synthetase purified to apparent homogeneity, was found by Fox and Kelley (1971) to consist of a single subunit of molecular weight 33,200, which underwent reversible association to aggregated forms composed of 2, 4, 8, 16 and 32 subunits in response to the addition of Pi, $MgATP^{2-}$ or Mg^{2+}. Similarly, *Salmonella* PRPP synthetase has a subunit molecular weight of 31,000 and the native enzyme exists in several states of aggregation with the predominant form having a molecular weight of about 160,000 (Switzer and Gibson, 1978). The enzyme from rat liver also has a complex molecular weight of subunit molecular weight of 40,500 enzyme molecules stacked together in long, linear aggregates as reported by Roth *et al.* (1974).

PRPP synthetase from *T.b.brucei* appears to be very similar in nature to the same enzyme from other sources possibly due to its tight regulation in order to satisfy the common requirement for PRPP of multiple branching metabolic pathways.

3.6. Conclusion.

The misunderstanding that existed long in the literature on the lack of the pentose phosphate pathway as part of the carbohydrate metabolic capacity of trypanosomes has finally been put right. It was during the period of this research that the presence of the enzymes of the classical pentose phosphate pathway was published by Cronin *et al.* (1989). The present work has confirmed that in bloodstream forms of *T.b.brucei* there functions an oxidative pentose phosphate pathway that forms a small, but significant, proportion of glucose metabolism.

The detection of PRPP synthetase activity in the bloodstream and cultured procyclic trypanosomes has also been of importance. The doubling time of the bloodstream forms of trypanosomes *in vivo* was used in combination with the RNA yield as estimated in Chapter 2 to calculate the specific activity of PRPP synthetase in relation to the cells requirement for RNA ribose synthesis (Table 3.27). The activity of PRPP synthetase in the crude extract of bloodstream forms appears not to be sufficient for RNA ribose synthesis and supply of PRPP for the trypanosomal phosphoribosyl pyrophosphate utilising salvage enzymes. Therefore, there need to be other sources of the carbohydrate moiety of RNA for synthesis, possibly through the action of phosphorylases and nucleoside kinases which would also yield the monophosphate blocks for RNA synthesis.

The properties of the trypanosomal PRPP synthetase studied in the partially purified preparation have been found to be similar to the properties of the same enzyme from other sources. The main differences have been the high substrate inhibition observed by $MgATP^{2-}$ of the enzyme from bloodstream forms of *T.b.brucei*, the inhibitory effect of excess (over ATP) concentration of magnesium and the lack of requirement of inorganic phosphate for stability during storage. Compartmentalisation of certain enzymes into intracellular organelles is considered as a peculiarity of trypanosomes. The cytosolic localisation of PRPP synthetase makes it possible for the R5P produced by the enzymes of the oxidative branch of the pentose phosphate pathway to be accessible to PRPP synthetase. It also requires the availability of hexose

Table 3.27. Calculation of specific activity of trypanosomal PRPP synthetase and requirement for nucleotide synthesis for replication.

The long slender bloodstream forms of *T.b.brucei* are the forms which multiply *in vivo* in rats. The observed doubling time of these organisms *in vivo* in rats is 6-8 hours (Balber, 1972).

The RNA yield of $4x10^9$ bloodstream forms of *T.b.brucei* was calculated as 782.12 ± 470.19 µg RNA (from 17 separate estimations) from the data obtained in the experiments presented in chapter 2.

Taking the average molecular weight of a monophosphate component of RNA as 339.45, and dividing the µg of RNA by this value, the rate of RNA synthesis in bloodstream forms of *T.b.brucei* is calculated to be approximately 1.37 nmoles RNA/min/10^9 cells, based on a doubling time of 7 hours.

The activity of PRPP synthetase in crude extracts of bloodstream forms of *T.b.brucei* is 0.625 nmoles PRPP formed/min/10^9 cells from data presented in chapter3.

It appears that there is insufficient PRPP synthetase activity in bloodstream forms of *T.b.brucei* to account for RNA ribose synthesis, and that there must also be other source(s) of the carbohydrate moiety of RNA for synthesis, such as phosphorylases and nucleoside kinases (Fish *et al.*, 1982a), which would also yield the monophosphate building-blocks.

phosphate in the cytosol either produced directly by the action of cytosolic hexokinase or transported to the cytosol through a transporter located on the glycosomal membrane.

3.7. Suggestions for future work.

Although the work described here seems to have no direct application to drug development, the detection, subcellular localisation and unfolding of some of the peculiar properties of trypanosomal PRPP synthetase such as high substrate inhibition by $MgATP^{2-}$, could prove useful to future workers in the search for safe and effective trypanocidal drugs.

It would be interesting to see if there is switching-on of the gene for PRPP synthetase when the availability of extracellular nucleic acid bases and nucleosides changes. This could be done by incubating bloodstream and procyclic forms of *T.b.brucei in vitro* in a defined medium with controlled additions of purine and pyrimidine nucleotide precursors and measuring the PRPP synthetase content to see if it changes. The PRPP synthetase gene may be part of an operon with the phosphoribosyltransferases, nucleoside kinases and other salvage enzymes constituents of the trypanosomes' complex nucleic acid metabolism.

Since trypanosomal PRPP synthetase is cytosolic it does not impose the additional problems of glycolytic enzymes for pharmacological inhibitors to get access to it and inhibit it. It might be possible to find an inhibitor for trypanosomal PRPP synthetase and so inhibit nucleotide synthesis due to inhibition of the salvage pathways which require PRPP.

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